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Life history and microbiome analysis of freshwater fingernail clams (sphaeriidae) exposed to trace metal pollution

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LIFE HISTORY AND MICROBIOME ANALYSIS OF FRESHWATER FINGERNAIL CLAMS (*SPHAERIIDAE*) EXPOSED TO TRACE METAL POLLUTION

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ABSTRACT

LIFE HISTORY AND MICROBIOME ANALYSIS OF FRESHWATER FINGERNAIL CLAMS (*SPHAERIIDAE*) EXPOSED TO TRACE METAL POLLUTION

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Aquatic habitats impacted by anthropogenic activities such as mining can contain metal mixtures of nonessential and essential trace metals. The consequences of chronic exposure to metal mixtures on the life history of benthic organisms are unclear, as are the potential effects on host-associated microbial communities. I use an energy-budget based methodology to ask whether exposure to metal mixtures influences the life histories of freshwater fingernail clams (*Sphaeriidae*) and if fingernail clams are selecting a different microbial community if chronically exposed to metal mixtures than if in a site that is not impacted by metal pollution. Fingernail clams are small, cosmopolitan, sedentary, ovoviparous bivalves found at sediment-water interfaces. Previous work indicates that reproductive output is correlated with adult size in benign environments. Sampling four impacted and reference lake, which is not impacted by metal pollution, I measured clam somatic growth (adult shell length), brood production, tissue Cd levels, RNA:DNA and the clam tissue and lake water microbial communities. I found that clams living in a benign lake display significantly larger shell length and greater reproductive effort than clams living along a lake polymetal gradient. Additionally, the five lake water microbial communities were indistinguishable but clams from each of the five populations

contained significantly different microbial communities. While I found the overall expected patterns amongst the life history data, I was not able to discriminate clearly amongst the four chain lakes with regards to bioenergetic partitioning, within-population clam growth and reproduction, and lake water microbial communities.

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INTRODUCTION

There are multiple, interacting threats to aquatic ecosystems, including pollution, climate change, and habitat loss (Sousa et al. 2008, Lopes-Lima et al. 2014, Bespalaya et al. 2018). Aquatic habitats tend to function as sinks for industrial and agricultural pollutants, particularly those habitats in which sediments and particulates bind to the pollutants, trapping them within water bodies (Callender 2003). The consequences of pollution in marine and estuarine habitats have been well-characterized, particularly with respect to interactions with global changes in water temperature, pH and oxygenation (for review, Rosenberg 1995, Sokolova and Lannig 2008). Comparable work has not been conducted in freshwater habitats, which have only recently been recognized as vulnerable to the interacting effects of pollution and global climate change (e.g. Ormerod et al. 2010). As indicated in a recent meta-analysis (Jackson et al. 2016), the consequences of interacting anthropogenic factors in freshwater habitats are different, and often opposite, from those documented in marine habitats, which indicates that we cannot simply extend conclusions from marine/estuarine work to freshwater systems.

There are a wide range of factors that impact freshwater habitats, which often vary over temporal and spatial scales. One widespread anthropogenic factor is trace metal contamination. Lead (Pb), zinc (Zn), cadmium (Cd), chromium (Cr), copper (Cu), and nickel (Ni) comprise a group of trace metals that have been heavily mined since the advent of twentieth century industrial mining and smelting operations (Callender 2003). Metal smelters are a major contributor to aquatic trace metal pollution worldwide, along with other industrial sources such as iron plants and wastewater dumping (Nriagu and Pacyna 1988).

Mining adds millions of tons of metals to the global biosphere each year, increasing metal circulation in the atmosphere, soil, and water (Nriagu and Pacyna 1988). Combined, these industries release metals directly to aquatic environments via tailing dumps, and indirectly via air pollution from smelting (Nriagu and Pacyna 1988, Callender 2003).

Pollution sourced from mining activities has been extensively documented . Mining activities deposit metals and other chemicals into sediments and water in marine, estuarine, and freshwater environments (Callender 2003, Marchand and Plumb 2005). As has been documented for decades, anthropogenic introduction of metals can disrupt aquatic ecosystems (Luoma 1983), with both industrial and atmospheric pollution adding metals to aquatic environments (Bryan 1971). Across all aquatic habitat types, the distribution of metals varies annually and seasonally due to alterations in flow, salinity, and adsorption (Luoma et al. 1990). Sediments, in particular, accumulate metals at high concentrations over long periods of time and are the most concentrated sources of metals in aquatic environments (Bryan 1971, Luoma 1983, Luoma et al. 1990).

A bioavailable metal is a metal that is present in the environment in a form that can be assimilated by organisms (Hare 1992). The most bioavailable form of a metal is the dissolved free metal ion, which has the fastest rate of uptake and accumulation in aquatic organisms (Lannig et al. 2008). Therefore, environmental conditions that lead to higher solubility of metal compounds (i.e., release from sediments) can facilitate higher concentrations of metal ions in the water column and increased levels of exposure. Metal bioavailability is affected by factors in the physical environment and in the organism. Aquatic factors that impact the concentration of ionic forms of metals include pH,

dissolved O_2 concentration, salinity, and amount of available organic particulates that act as ligands (Luoma 1983, Bryan and Langston 1992, Rainbow 2007, Li et al. 2013). In general, the bioavailability of trace metals increases when dissolved organic compounds that bind them are in low concentration, temperature is elevated, pH is lowered, hardness (Ca^{2+} and Mg^{2+}) is lower, and/or dissolved O_2 is low. Overlying features of the habitat, including seasonality, water type, and features of the organisms themselves influence metal uptake dynamics (Luoma 1983, Hare 1992).

Trace metals, particularly divalent cations (e.g., Cd^{2+}, Pb^{2+}), can enter animal tissues such as respiratory epithelia through cation transporters that otherwise would transport the calcium and/or magnesium ions that are essential for cell function (Ballatori 2002). Essential metals that normally are involved in intracellular transport and enzymatic activity, such as Zn and Fe, overwhelm metabolic pathways when present at higher than necessary concentrations; nonessential metals (Pb, Cd, Ag, Hg) can have detrimental effects at relatively low concentrations. Trace metal toxicity thresholds vary across aquatic organisms depending on the animal, the metal, and the environmental conditions. Absorption of excess amounts of essential metals and/or any quantity of nonessential metals can have consequences for glycolysis, oxidative phosphorylation, the Krebs cycle, and the metabolism of amino acids, carbohydrates, and lipids (e.g., Bryan 1971, Sokolova 2004, Strydom et al. 2007, Yang et al. 2015, Pirone et al. 2019).

Bivalves as model system

Benthic organisms are often used as biomonitors because of their reduced mobility and close proximity to pollutants that are retained in sediments. 'Whole-sediment' toxicity testing on benthic organisms is a standard practice in ecotoxicology

(Monserrat et al. 2007, Besser et al. 2015). Benthic organisms can accumulate metals in their tissues, facilitating transfer of metals up the food chain (e.g., MacCrimmon 1982, Croteau et al. 2005, Mathews and Fisher 2008). Of the wide array of benthic taxa, bivalves are often used as bioindicators for pollution in marine, estuarine, and freshwater habitats because they are sedentary, can be present in large numbers, and are easily maintained in the lab for acute and chronic tests. Bivalves are particularly vulnerable to metal exposure because they can take up metals via absorption across their gills, through contact with the sediment, and through digestion of contaminated food such as algae filtered from the water or detritus from the sediment surface. Several factors influence metal accumulation rates in bivalves, including season, animal size, changes in life history, environmental hydrodynamics, and metal bioavailability (Luoma 1983, Cain and Luoma 1986, Cain and Luoma 1990, Boening 1999, Otchere 2003, Perceval et al. 2004, Lesser et al. 2010).

In freshwater habitats, the "fingernail" clams (*Sphaeriidae*) are a dominant and well-studied taxon that exhibit many traits that make them useful as indicators of environmental quality. Fingernail clams are benthic filter feeders found in permanent and temporary habitats, including rivers, ponds, lakes, streams, and ditches (Martin 1998, Sloss et al. 1998, Smith and Beauchamp 2000, Mackie 2007). Within these zones they reside at the surface, in the water column, in weedbeds, and in the sand and mud. They are a common food sources for fish and birds (Hickey et al. 1999, Mackie 2007). Fingernail clams are sedentary filter feeders (Martin 1998) taking in detritus, algae, bacteria, and associated contaminants from both the sediment and water column (e.g. Doherty 1990, Hickey et al. 1999). Fingernail clams have a patchily abundant distribution

(Sloss et al. 1998) and four genera of fingernail clams, *Corbicula, Sphaerium, Musculium,* and *Pisidium*, share similar physiological and life history traits and often coexist (Mackie 2007). Fingernail clams are hermaphroditic and ovoviviparous; the young are developed internally within brood pouches located in the gills of the parent clam before being released, shelled, to the environment (Mackie 1978b, Martin 1998, Kullman et al. 2007). At release, the young are "fully formed miniature adults" (Smith and Beauchamp 2000); because of this reproductive strategy, the reproductive output of fingernail clams is easier to measure (Mackie 1978b, Sandusky 1983) compared to molluscs that use the broadcast spawning of sperm and eggs to reproduce, which is challenging to quantify. Reproductive output is a reliable indicator of overall environmental quality because a number of factors including temperature, dissolved O_2, and environmental pollution levels alter brood characteristics (Mackie 1978c, Joyner-Matos et al. 2007, Joyner-Matos et al. 2011).

The best-studied genus of freshwater clam, *Corbicula*, is an established model species for ecotoxicology, likely because it is an invasive species and is the largest fingernail clam (Luoma 1983, Doherty 1990, Santos et al. 2007, Sousa et al. 2009). The other fingernail clam genera are not as well-studied within the context of ecotoxicology, although some work has been done to document the potential of them as a bioindicator species (Mackie 1978a, Schoonover et al. 2016).

Energy budget and pollution

All organisms have an energy budget, meaning they must balance energy acquisition with investment in processes related to daily function and fitness (Sokolova et al. 2012). In freshwater clams, metabolic storage is measured as net energy reserve

(typically glycogen content), reproduction and development are evaluated using brood characteristics specific to each taxon, growth typically is measured via shell size (using a series of mathematical parameters) and/or tissue mass, activity is measured by movement including burrowing and climbing, and somatic maintenance can be evaluated in multiple ways including metabolic profiling (Cherkasov et al. 2006, Kooijman 2009, Sokolova et al. 2012, Yang et al. 2015, Cheng et al. 2018). Metabolic changes in response to stress are associated with overall effects on growth and reproduction via reduced energy reserves (Yeung et al. 2016), such as reduction in storage of carbohydrates and/or lipids. An increased demand for energy in maintenance and repair processes can scale up to detectable changes in population density, biomass, and structure in both fish and bivalves (e.g. Perceval et al. 2004).

Exposure to stressors, including trace metals, can result in predictable energetic response patterns depending on the magnitude of the stress (Sokolova et al. 2012). A mild stress causes a small disruption from homeostasis, exhibited as the diversion of some metabolic energy from reproduction to maintenance and repair processes. This can be detected as decreased reproductive success. If a mild stress becomes a moderate stress, most energy from both reproduction and growth will be redirected to maintenance and repair, exhibited by an increased demand for ATP by maintenance processes such as circulation, acid-base regulation, and protein production. This redirection of energy in fingernail clams would manifest as a reduced brood size and reduced shell length (if chronic) or reduced wet mass (if acute). Under highly stressful conditions, all available energy will be diverted to maintenance in attempt to survive. This is not compatible with population persistence (Sokolova et al. 2012).

The RNA:DNA (ratio) is a biochemical marker representing the rate of organismal protein synthesis (Lannig et al. 2006, Lesser et al. 2010). In several marine organisms, including bivalves, the RNA:DNA indicates overall metabolic condition, short term growth, nutritional status, and response to physiological stress (Revankar and Shyama 2009, Lesser et al. 2010, Sanders et al. 2013). The ratio changes rapidly in response to nutritional changes and environmental stressors (Tsangaris et al. 2010). Protein synthesis is expected to increase in response to moderate exposure to pollution as the organism increases production of cellular protective proteins (e.g. Tsangaris et al. 2010). Exposure to high levels of pollution can suppress protein synthesis; this metabolic disturbance is reflected as lower ratios (Tsangaris et al. 2011). Variations in RNA:DNA ratios in organismal tissue correlate to exposure to sublethal doses of contaminants (Revankar and Shyama 2009, Tsangaris et al. 2010).

Understanding the consequences of metal pollution at the organismal and community wide levels requires a system in which pollution response can be measured by detectable energy budget reallocation in individuals. This is most successful when measurements can be taken at multiple levels of organization, from the cellular (e.g., RNA:DNA) to the organismal (e.g., reproductive output). Ultimately, these individual, physiological traits should be linked to population-level metrics, such as size/frequency distributions or patterns in population-level reproductive timing and output to characterize the potential effects of the pollutant (e.g. Sousa et al. 2008, Mouthon 2009).

The Microbiome

One potential contributor and/or cost to energy budget allocation within the context of stress physiology and/or ecotoxicology that has received relatively little

attention is the relationship between eukaryotic hosts and microbes. All animal species harbor microbial communities, referred to as their microbiome which often influence the fitness of their hosts (McKenzie et al. 2012, McFall-Ngai et al. 2013, Becker et al. 2015, Zaneveld et al. 2017). Microbial contributions to eukaryotic systems can encompass a wide range of mechanisms, including but not limited to directly and indirectly modulating host nutrient acquisition, tolerance of abiotic factors, protection from pathogens, and reproduction (Dubilier et al. 2008, Carey and Duddleston 2014, Bahrndorff et al. 2016, Abele et al. 2017, Antwis 2020).

The bivalve-associated microbiome has been studied for almost a century in species relevant to the shellfish industry (Lokmer and Mathias Wegner 2015). Because they are predominantly filter-feeders, marine bivalves accumulate a rich and diverse community of bacteria (Zannella et al. 2017). Until recently, these studies focused on three main groups of shellfish bacteria: bacteria naturally found in marine and estuarine habitats, bacteria associated with human fecal contamination, and bacteria associated with improper food handling (Anacleto et al. 2013). Documenting the bivalve microbiome can be challenging as microbial profiles vary from individual to individual, even within the same habitat (Anacleto et al. 2013). Bivalves interact with potential symbionts across their epithelia, particularly the gills, which are used for gas exchange and feeding (Bright and Bulgheresi 2010). Bacteria also have been isolated from the foot, gill siphon, and digestive glands of marine bivalves (King et al. 2012, Baldi et al. 2013, Lokmer and Mathias Wegner 2015, Arfken et al. 2017, Lim et al. 2019).

There is a slowly-growing body of literature on marine bivalve microbiomes and how they shift over time in response to pollution; two initial studies suggested that

marine bivalves may be selecting for microbial partners that contribute to pollution tolerance. A 2013 study on Manila clams (*Ruditapes philippinarum*) from polluted Italian lagoons isolated 14 strains of Hg-resistant bacteria from the clam siphon, gill, and hepatopancreas (Baldi et al. 2013). A 2018 study of Manila clams identified 19 core microbiome taxa in the hepatopancreas microbiome community and compared these communities across sites with different levels of pollution (Milan et al. 2018). They found a correlation between shifts in the bacterial community and changes in microbial mRNA transcripts associated with xenobiotic degradation pathways, indicating that the bacteria were interacting with the environmental pollutants while associated with their hosts (Milan et al. 2018).

As factors driving the selection of microbial partners by bivalve hosts is virtually unknown (outside of hydrothermal vent habitats), we can look to recent investigations of another host that is currently facing an environmental challenge: amphibians that are exposed to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al. 1999, McKenzie et al. 2012, Scheele et al. 2019, Kruger 2020). In order to investigate whether amphibians were preferentially selecting microbes with antifungal capabilities from their environment, researchers compared the amphibian skin microbial community to the microbes of their surrounding habitats (McKenzie et al. 2012, Walke et al. 2014, Kruger 2020). Broadly, they found discrepancies between the external skin of the amphibians and their environment, suggesting the presence of a host-specific selection mechanism for microbes producing anti-*Bd* metabolites. These amphibian studies could serve as models for tests of whether freshwater invertebrates like fingernail clams that are in constant contact with pollutants may select microbial partners on the basis of their

tolerance to the pollutant. Unfortunately, the fingernail clam microbiome has not, to our knowledge, been characterized, and therefore we cannot yet assess whether the freshwater clam microbiome reflects habitat characteristics like the presence of a pollutant (or a pathogen).

Study Overview, Study Site, and Hypotheses

The proposed project combines the lessons learned from marine bivalve physiology and microbiology with techniques from freshwater amphibian microbial studies. I explore trace metal pollution as the stressor potentially driving the formation of clam-microbial relationships, akin to the fungal pathogen stressor in amphibian studies and anthropogenic pollution stressors in marine bivalve studies.

I explored the responses of populations of fingernail clams from a series of "chain" lakes in the Coeur d'Alene (CDA) River basin that is downstream from an Environmental Protection Agency (EPA) Superfund site in northern Idaho. The bodies of water within the floodplain of the CDA River contain consistent stressors that affect bioenergetics of organisms, such as Cd (Sprenke et al. 2000, Bookstrom 2013, Higbee 2017). The characteristics of these lakes were detailed in the thesis of a previous graduate student in my lab (Chantilly Higbee) who conducted monthly samples of triplicate sites in 11 of the chain lakes. She showed that sites within a given lake are indistinguishable within a sampling period, and that the limnology of each lake varies seasonally (Higbee 2017). She also confirmed the presence of fingernail clams in the lakes. As her data confirm, a subset of the lakes can be considered to represent a gradient of likely trace metal exposure due to their concentrations of dissolved metal levels. These chain lakes represent the type of gradients that have been used successfully to study both microbial

species (Carey and Duddleston 2014) and fingernail clam physiological responses to pollution (Baudrimont et al. 1999).

A century of mining activity resulted in the deposition of over 56 million tons of metal-containing tailings downstream from the Bunker Hill Superfund Site (Sprenke et al. 2000, Balistrieri et al. 2010). A large mining and smelting industry flourished in northern Idaho from the 1800's to the closing of the lead mine in 1983 (EPA, 1999) The chronic dumping resulted in high, variable levels of trace metals in the surrounding areas (Sprenke et al. 2000, Balistrieri et al. 2010). Large quantities of mine waste were carried down the CDA River and into Lake CDA. Although the CDA River is monitored by the United States Geological Service (Bookstrom 2013), the "chain" lakes which lie within the river's floodplain are not. There have been several assessments of the macroinvertebrate community responses in this system, some which confirm the transfer of metals to other organisms in the food web, from invertebrates to fish and even swans (Blus et al. 1991, Hoiland et al. 1994, Farag et al. 1998, Farag et al. 1999, Farag et al. 2000, Mebane 2003a).

I collected data sets from four chain lakes of the CDA River that represent a gradient of metal pollution and from one reference lake in a neighboring watershed that has not been impacted by mining pollution but which is regularly used as a comparison site to the chain lakes (Blus et al. 1991, Farag et al. 1998).

Data sets:

1) lake limnology (water temperature, conductivity, pH, dissolved O_2, alkalinity) and "metal exposure" (total metal levels, clam tissue metal content)

2) two aspects of bioenergetics: reproductive output (brood size) relative to somatic growth (shell length), and whole-clam investment in protein synthesis (RNA:DNA)

3) the surface water bacterial community

4) the whole-clam bacterial community

Hypotheses

(1) I hypothesize that the five clam populations will exhibit different life history strategies depending upon their exposure to metals. The "level of exposure" will be estimated from the lake water total metal levels and the clam tissue metal burden. Specifically, I predict that clams from more polluted lakes will exhibit lower overall somatic growth and lower reproductive output relative to somatic growth than will clams from the reference (unpolluted) lake or lakes with lower metal concentrations.

(2) I hypothesize that the five clam populations will exhibit different investment in whole-organism energetic partitioning. Specifically, I predict a decreased investment in protein synthesis (RNA:DNA) in clams from habitats that are more impacted by metals.

(3) I hypothesize that the microbial communities will differ among the lakes.

(4) I hypothesize that the microbial community of each clam population will differ from the environmentally available microbes.

METHODS

Field sampling

Sampling locations were selected based on previous work in the Joyner-Matos lab, some of which was from Cody Schoonover's work (Schoonover 2013, Schoonover et al. 2016) and most from Chantilly Higbee's thesis (Higbee 2017). All clam and water collections were done in one day (August 7, 2020) and sites were visited in the order

listed. They were sampled in the same order as in previous lab work (Higbee 2017) to replicate methods. Permission was granted from the Coeur d'Alene Tribe of Indians to sample from the surface waters of Benewah L., which lies within reservation boundaries; permission was not required at the other sites as all were visited at public access sites and the State of Idaho does not require sampling permits for invertebrates. For methods that were new to the Joyner-Matos lab and/or not described in protocol form in previous theses (e.g., clam dissection, DNA extraction), detailed protocols are supplied in the Appendix. An overview map of the sampling locations is in Figure 1 and detailed maps and photos in Figures 2-6.

At Rose L. (Figure 2), adjacent to the fishing dock, clams were sampled at the edge of submerged vegetation, which was predominantly cattails, and from submerged vegetation in open lake water. At Bull Run L. (Figure 3) clams were sampled from aquatic vegetation along the bank of the lake where it is adjacent to W Bull Run Rd.; the lake water appeared to have an oily sheen and texture. The sampling site at Medicine L. (Figure 4) was on the peninsula adjacent to the parking area. Clams could only be collected along the southern margin of the peninsula, from pockets of water in between dense thickets of emergent vegetation. At Thompson L. (Figure 5) clams were found close to the lake bank, and associated with emergent aquatic vegetation in an area with slightly higher water flow. At Benewah L., the reference site (Figure 6), clams were sampled from the sediment-water interface at the shoreline, near aquatic vegetation.

At all sites, clams were collected using the same methods. Clams were collected via a dipnet or sieve that was swept along the vegetation near the sediment/water interface; care was taken to minimize disturbance of the sediment, especially in the

metal-polluted lakes. Once collected, clams were rinsed briefly in lake water and then placed individually into 8 cm long pieces of PVC pipe (2.5 cm internal diameter) that were covered on each end with 0.5 mm plastic mesh that was secured with zip ties. This design ensured that any juveniles extruded by the clams would stay with the adult and it allowed for sufficient water flow through the cage. The cages were placed into 1.5 gallon Ziploc bags that were filled with surface water from the collection site to provide algae and to maintain water quality characteristics of each lake. The Ziplocs were then double-bagged and stored in coolers that had ice packs. At the end of the field day, ice packs were replaced with new packs and the coolers were stored outside overnight. The next day the coolers were taken to EWU and new ice packs were placed in each cooler. Clam dissection and processing began at this point.

Water sampling

Limnological variables (conductivity, temperature, dissolved O_2, pH) were taken using a YSI Professional Plus meter. These measurements were taken in triplicate, closely spaced, from a location within 1 m of where clam sampling occurred. The triplicate measurements were averaged to generate a single estimate of each water quality parameter per lake.

Given the disturbance to the sediment that occurred during clam collection activities, lake water samples were collected at sites up to 2 m away from clam collection, but at comparable water depths, vegetation types and distance to the shoreline from the clam collection sites. Water cannot be collected prior to clam sampling as fingernail clams are patchily abundant. Water was collected into two types of containers. Water to be used for trace metal analysis was collected into 10% nitric acid-washed 500

ml plastic bottles. A field blank containing Milli-Q (ultrapure) water was prepared in the same type of bottle, transported to the field, and processed in the same manner as the lake water samples. Water to be used for alkalinity analysis was collected into 125 mL plastic bottles. For lake water microbiome, five 50 mL samples of water were collected into individual 50 mL Falcon tubes. The only exception was at Bull Run L., where only four 50 mL samples were taken at the sampling site itself due to a counting error; the remaining fifth 50 mL tube was filled immediately upon reaching EWU from an extra container of lake water sampled. This container of water was not used for transporting clams.

Water samples were transported in coolers with ice packs, in the same way as clam samples, back to EWU. Microbiome water samples were transferred to -80°C freezer for long term storage until DNA extraction. For metal analysis, all lake water samples were processed using methods previously used in the Joyner-Matos lab (Higbee 2017) and modified from protocols from Dr. Carmen Nezat (EWU Geology) and stored at 4°C until processed (see clam tissue and lake water metal analysis section).

Approximately 36 hours after the lake water samples were brought to EWU, lake water alkalinity was determined through titration at room temperature, following standard procedures (Wetzel and Likens, 1991). This analysis generated a single alkalinity estimate per lake.

Clam dissections

All clams were dissected within 36 hours of arrival at EWU. Clams remained in their original cages in their original bags of lake water, undisturbed in coolers until they

were dissected. Clams were not aerated, fed, or exposed to new water for the duration of that time. No clams died.

Clams selected for microbiome processing were chosen randomly from the bags until I reached n = 6 for each lake. Briefly, individual clams were removed from their cage, any extruded brood in the cage were counted as part of the individual total reproductive effort. Clams were rinsed in autoclaved deionized water to remove detritus; shell length was measured from adductor to adductor (Figure 7) to the nearest millimeter using calipers. Clams were then dissected using sterile methods that I developed. Briefly, clams were opened and the pieces of shell were removed from the dissection area. Next, the shelled larvae in the mantle sac and brooded larvae from brood sacs in the gills were counted and removed. The number of shelled and brooded larvae were added to the number of extruded juveniles, if any, to quantify the total brood count for each clam (brood types illustrated in Figure 7). The whole clam body (digestive gland, foot, gills, brood sac tissue) was then transferred to a sterile 1.5 mL tube for long term storage at -80°C. For each lake, an empty dissection control tube was created midway through the microbial dissections; these were sterile tubes that I touched with sterile dissection tools, mimicking the placement of tissue inside the tube. After reaching n = 6 microbiome clams per lake, the remainder of the clams were processed for tissue analyses. Dissection proceeded in a mechanically identical fashion but using clean instead of sterile techniques. The shell length and total brood count of each individual clam was recorded, but clams were pooled three clams per tube rather than one clam per tube.

Patterns in adult shell length of all dissected clams were compared across the five clam populations with a Kruskal-Wallis ANOVA, a non-parametric ANOVA, because

the data violated the assumption of normality (Shapiro-Wilk normality test); the Kruskal-Wallis ANOVA uses the Dunn's Method of post-hoc comparisons. Reproductive output, which is the total brood count (sum of extruded juveniles, shelled larvae and brooded larvae) divided by the adult clam shell length, was compared across the five clam populations with all clams included, regardless of brooding status, using Kruskal-Wallis ANOVA. As total brood count is expected to scale to adult size in a clam population that is living in optimal conditions, I analyzed the relationship between total brood count and adult shell length, using data only from those clams that were brooding. Because these data violated the assumptions of constant variance and/or normality, the data were square-root transformed prior to analysis by linear regression. With this transformation, I could assess this relationship collectively across the five populations and separately for the Bull Run L., Thompson L., Rose L, and Benewah L. populations but not Medicine L. as the data continued to violate assumptions. All statistical analyses were conducted using SigmaPlot ver. 11.0.

Clam tissue and lake water metal analysis

Clam tissue metal burden and total lake water metal levels were quantified using methods previously used in the Joyner-Matos lab (Higbee 2017). Briefly, lake water samples and the field blank were kept in the coolers with the bags of clam cages until they arrived at EWU. A volume of 2.5 ml was removed from each 500 mL sample and replaced with 2.5 ml of 70% trace metal grade nitric acid. Acidified samples were stored at 4°C until processed. Because the samples were acidified before they were filtered, these samples provide estimates of the total metal content (dissolved and suspended, which should be higher than bioavailable metal concentrations) because the acidification

removed metal ions from the suspended particulates. Prior to analysis, 15 ml samples from each lake were filtered through Whatman Puradisc 0.45 µm PTFE filters and stored at room temperature until analyzed by Dr. Nezat on an inductively coupled plasma atomic emission spectrometer (ICP-OES). As lake water samples were taken from the same site and at only one sampling time, the lake water metal content and hardness are presented but not entered into a statistical analysis. To calculate water hardness, the ICP-OES values for lake water calcium and magnesium content (mg/L as Ca^{2+} or Mg^{2+}) were entered into the following equation: Total hardness (mg/L as $CaCO_3$) = 2.5 * [calcium content] + 4.12 * [magnesium content].

Pooled clam samples (three clams per sample were pooled post dissection) were digested in 30% trace metal grade hydrogen peroxide at 100-125°C using methods previously optimized in the Joyner-Matos lab (Higbee 2017). Briefly, samples were digested to a fine paste, solubilized in 2% nitric acid and then then filtered through Whatman Puradisc 0.45 µm PTFE filters with between 9-10 mL of 2% nitric acid. Three tissue-free samples (methods blanks) were interspersed with the tissue samples to provide digestion blanks. Samples were stored at 4°C until ICP-OES analysis by Dr. Nezat.

Certified reference materials for the analysis included high purity standards Trace Metals in Drinking Water Samples and Soil Solution B. Samples were loaded into the ICP-OES randomly. The following elements were quantified: As, Ca, Cd, Mg, Pb, Zn. Prior to statistical analysis, values were evaluated with respect to the limit of detection (LOD) for that element for that day's run on the instrument; LOD and LOQ (limit of quantitation) values for the lake water and tissue analysis runs are presented in Table 4. When the concentration of an element fell below the LOD for a subset of samples, a ½ *

LOD was reported for that element in statistical analyses (Zhang 2007). As the clam tissue As levels for all samples were < LOD, these data are not presented. Pb data on tissues could not be collected because of spectral interferences, including a peak in a method blank. Given the presence of Zn in the digestion blanks, only those clam samples that were higher than the digestion blanks are presented; given the small sample size, we did not analyze these data. Clam tissue Cd content was compared across the five populations using ANOVA. I used Spearman Rank Order Correlation (SigmaPlot ver. 11.0) to test for relationships between tissue Cd, averaged shell length of the three clams in each pooled sample, and averaged total brood count of the three clams in each pooled sample. After Bonferroni correction of the acceptable alpha error rate, only one correlation was significant (tissue Cd and shell length). To explore that relationship further, I used a linear regression following natural log transformation.

Nucleic acid extraction

Nucleic acids were extracted from clam and water samples using the Qiagen DNeasy Blood and Tissue Kit. As this method was used to extract DNA for the microbiome characterization, this description focuses on DNA. In the next section I describe how I measured RNA:DNA in these extractions. Clam and lake water DNA extraction controls (tubes that received a swab that had sampled sterile MGW that received the same kit elements as the sample tubes) and clam dissection controls (tubes that were touched by dissection tools during the sterile clam dissection process) were processed at the same time. Briefly, individual clam and dissection control samples were removed from the -80°C freezer and processed immediately using the Qiagen kit protocols. The final DNA product was stored at -20°C until downstream PCR. The water

samples were put through two full freeze-thaw cycles to ensure greatest sample heterogeneity, accounting for freeze/thaw cycles that occurred during methods optimization. The water samples were removed from the -80°C and thawed at 4°C for 36-48 hours until all ice crystals had disappeared, then inverted to mix and placed back in the -80°C freezer until fully frozen, and then thawed again in the same conditions. Immediately after reaching the end of their second thaw, the water samples and the lake water controls were swabbed using modified methods from Walke et al. (2014). The whole-water-sample DNA from the water on the swabs was then extracted using the DNeasy Blood and Tissue Kit, following the manufacturer's protocol for Gram-positive bacteria, which includes a lysozyme pre-treatment. DNA was eluted in 100µl sterile molecular grade water. The final DNA product of each clam and water sample was determined using the Qubit fluorometer (Invitrogen) in the L. Matos lab before storage at -20°C until downstream PCR.

RNA:DNA

The RNA to DNA ratio was measured via the Qubit 2.0 Fluorometer on every clam tissue sample that was processed for microbiome analysis (n = 6 per lake). Briefly, the RNA and DNA content of the final product of the Qiagen kit were measured via the Invitrogen Qubit RNA HS Assay Kit and Invitrogen Qubit dsDNA BR Assay Kit, respectively, following the manufacturer's instructions. For each sample, the ratio was calculated by dividing the total RNA (in ug/mL) by the total DNA content (in ug/mL). The RNA:DNA was compared across the five populations using a Kruskal-Wallis ANOVA. I used Spearman Rank Order correlation (SigmaPlot ver. 11.0) to test whether RNA:DNA was correlated with shell length and reproductive characteristics.

Polymerase Chain Reaction

PCR for microbial community characterization through Illumina sequencing was conducted according to the protocol from the Walke lab, an Illumina MiSeq Sequencing protocol modified from the Earth Microbiome Project (Thompson et al. 2017).

PCR for Illumina MiSeq sequencing was conducted using the DNA from each of the 30 clam samples, 25 water samples, and the pooled control sample according to a modified version of the Earth Microbiome Project's 16S Illumina amplicon protocol (Caporaso et al. 2012, Thompson et al. 2017). These samples were amplified using universal primers (barcoded 515F and 926R) (Quince et al. 2011, Parada et al. 2016), which target the V4-V5 region of the 16S rRNA gene (Quince et al. 2011). The primer pair for each sample contained a unique barcode sequence on the forward primers that allows for identification of the PCR product in the (downstream) pooled sample. PCR was performed in triplicate with a negative (no template) control per sample. PCR took place in the Walke lab, using aseptic techniques (see Appendix). Post-PCR, amplified DNA fragments of the individual samples and their negative controls were checked via agarose gel electrophoresis. None of the PCR negative controls contained detectable bands in the gel. After DNA quantification of amplicons using the Qubit 4.0 Fluorometer, individual samples were pooled at equimolar concentrations into a single. 100µl of the pooled sampled was cleaned using the Qiagen QIAquick PCR Purification Kit. Samples were sent to the Dana Farber Cancer Institute for 250 base pair (bp) single-end sequencing on the Illumina MiSeq platform (Caporaso et al. 2012).

Following an initial set of reactions, in which the DNA extraction control showed potential contamination, all controls (dissection, clam tissue, water) were run on a

1492R/8F PCR to determine which samples, if any, were contaminated; PCR with these primers identifies the presence of bacterial DNA. I identified five contaminated DNA extraction control samples (three water and two clam extraction control samples, Table 1). These five samples were pooled by combining 10µl from each of the contaminated DNA extraction control samples (Table 1) following Illumina PCR to create a "control" sample that also was sequenced. The 43 bacterial features that were in the pooled control sample were later bioinformatically filtered out of the 55 clam and water samples.

Microbiome characterization

Microbiome analysis was conducted using the next-generation bioinformatics platform QIIME2 (Quantitative Insights Into Microbial Ecology, version 2021.4.0) (Bolyen et al. 2019). Specifically, sequences were demultiplexed, scored and filtered for quality using the tool Deblur (q2-deblur) (Amir et al. 2017). Taxonomy was assigned using the q2-feature classifier (Bokulich et al. 2018) with the -sklearn Bayes taxonomy classifier, using a pre-trained Silva database (version 138.1) for the 515F-926R primer set. After filtration, the total number of features (defined genomic region with some known, annotated function) from all samples (clam tissue, water, control) was 1,514. Clam and water samples ranged from 423 to 6,508 and 12,033 to 27,372 sequences per sample, respectively. After removal of the 43 feature sequences that were present in the pooled extraction control from all clam and water samples, the clam and water samples ranged from 233 to 4,600 and 5,535 to 19,367 sequences per sample, respectively. A phylogenetic tree was constructed using fasttree2 (Price et al. 2010). To determine a sampling depth that would capture the bacterial diversity in both the clam tissue and water samples, samples were rarefied from 500 to 5,000 sequences per sample in

increments of 500 sequences and visualized to identify the sequencing depth at which these diversity measurements leveled off. Ultimately, analysis rarefication depth was set to 700 sequences per sample, meaning samples with fewer than 700 sequences were not retained in the analyses, resulting in the removal of two clam tissue samples (one from Rose L. and one from Thompson L.).

I evaluated the effects of eight categorical and numerical variables on bacterial community structure. Categorical variables included sample type (clam tissue or water), site (lake), drainage type (Coeur d'Alene or St. Joe, to represent the presence or absence of metal pollution, respectively), and clam tissue Cd. Clam tissue Cd was a categorical variable noting if the clams were sampled from Thompson L. or Medicine L., which had significantly higher clam tissue Cd concentrations than clams from the reference lake (see Results). To distinguish between clam tissue and water samples at a given site, a final categorical variable of "sample type + sample site" was created in order to analyze the clam and water samples from each lake as separate groups, as the variable "site" did not treat clam tissue and lake water samples from a given lake as separate types of sample. The numerical variables included clam shell length in mm, clam total brood count, and clam reproductive output (brood count divided by adult clam shell length).

Metrics of alpha diversity summarize the richness (number of taxa present), evenness (distribution of taxa), or both (Willis 2019). Alpha diversity was assessed using the following metrics (Pearson et al. 2019, Campos 2020) using q2-diversity: observed features (synonymous with Operational Taxonomical Units or OTUs, with 100% sequence similarity), a measure of richness; Pielou's Evenness (Pielou 1966), a measure of within-sample community evenness; Faith's phylogenetic diversity (Faith 1992), a

measure of richness with respect to phylogenetic relationships between the features; and Shannon's diversity index, a measure of richness and evenness. Because these metrics were not normally distributed, the effects of the categorical variables were analyzed using the Kruskal-Wallis test. For the continuous (numerical) variables, a Spearman correlation was used. Categorical alpha diversity metrics were visualized using box and whisker plots; numerical alpha diversity metrics were visualized using scatter plots (data not shown). To visualize the overlap between clams tissue and water features, features present in all samples were obtained using q2-feature-table and visualized in a Venn diagram using the program Venny (Oliveros 2007-2015).

Beta diversity is a measure of the degree of difference in community composition between two sets of samples (Ursell et al. 2012). Beta diversity was assessed using the following metrics (Pearson et al. 2019, Campos 2020) using q2-diversity: the Bray-Curtis measure of dissimilarity, which reports differences in both presence or absence and abundance of features; Jaccard similarity, which reports differences in the presence or absence of features; unweighted UniFrac distance (Lozupone et al. 2007), which reports differences in the presence or absence of features in the context of a phylogenetic tree (evolutionarily related sequences); and weighted UniFrac distance (Lozupone and Knight 2005), which reports differences in the presence or absence and abundance of features in the context of a phylogenetic tree (Pearson et al. 2019). Differences in beta diversity metrics among categorical variables were analyzed using a permutational multivariate analysis of variance (PERMANOVA).

Principal Coordinates Analysis (PCoA) ordination was used with these four measures of beta diversity to visualize the differences among groups, using the

categorical variable “sample type” to identify the differences between clam tissue and water samples. In addition, the categorical variable “sample type + site” was visualized to distinguish the effects of sample type from those of location. PCoA plots were generated and visualized using Emperor (Vázquez-Baeza et al. 2013). Taxa barplots showing relative abundances of bacterial taxa were generated using -taxa barplot and visualized using view.quiime2.org.

RESULTS

Limnology

Limnological variables for each lake that were collected via the YSI (pH, temperature, dissolved O_2, conductivity), as well as alkalinity and hardness are reported in Table 2. Lake water total metal levels are in Table 3; the LOD and LOQ values and the samples that fell below them are listed in Table 4.

Clam shell length and brood size

All clams that were collected from each lake were dissected. While I did not find any dead clams, one shell was completely empty and was not included in calculations. A summary of shell length and brood characteristics is in Table 5.

The clam shell lengths ranged from 2.5 - 11 mm. Clams from Benewah L. had significantly longer shells than did clams from any other lake (Kruskal-Wallis ANOVA, $p < 0.001$; multiple comparisons, $p < 0.05$). Clams from Bull Run L. had significantly longer shells than did clams from any of the other chain lakes ($p < 0.05$).

The total brood (sum of all three types of offspring) of the clams across all lakes ranged from 0 to 29 (Table 5). As comparisons in total brood across clams of different sizes is not valid, I compared reproductive output (total brood divided by adult shell

length) across the five populations (Table 5, Figure 9); these analyses included all clams. Clams from Benewah L., all of which contained brood, had a significantly higher reproductive output than did clams from any of the chain lakes (Kruskal-Wallis ANOVA, $p < 0.001$; multiple comparisons, $p < 0.05$). Clams from all other chain lakes had a significantly higher reproductive output than did clams from Rose L. ($p < 0.05$).

Considering only those clams that were brooding, across all five populations, the total brood increased significantly with shell length ($R^2 = 0.48$, $p < 0.001$; untransformed data presented in Figure 10). The relationship between total brood and shell length varied by lake population (Figure 11). Even with the square root transformation, this relationship did not meet the assumptions of linear regression for Medicine L. clams (data not shown) and was not a significant relationship for Rose L. clams ($p = 0.4$). The relationship between total brood and shell length was positive in the remaining three populations, but the slope of the regression lines were indistinguishable across the three populations (Bull Run L., slope = 2.16, $R^2 = 0.152$, $p = 0.003$; Thompson L., slope = 2.36, $R^2 = 0.245$, $p = 0.009$; Benewah L., slope = 1.64, $R^2 = 0.285$, $p < 0.001$).

Clam tissue metals

Tissue Cd levels were significantly higher in clams from Medicine L. than in those from Benewah L. and Rose L. (Kruskal-Wallis ANOVA, $p <0.001$, comparisons, $p < 0.05$) (Table 6). Clams from Thompson L. also had higher tissue Cd levels than did those from Benewah L. ($p < 0.05$). In the Spearman Rank Order correlation, tissue Cd was significantly and inversely related to the average shell length of the clams in each pooled sample ($r_s = -0.665$, $p = 0.00036$; Figure 13a). Tissue Cd also was inversely related to average total brood count ($r_s = -0.449$, $p = 0.027$), but not to average

reproductive output (r_s = -0.349, p = 0.094). In the linear regression of natural log-transformed data, tissue Cd was negatively related to shell length (slope, -3.595; R^2 = 0.38, p = 0.001; Figure 13b). However, when Benewah L. clams were removed from this analysis, the significant relationships disappeared.

RNA:DNA

The RNA:DNA ratio did not differ across the five clam populations (Kruskal-Wallis ANOVA, p = 0.734; Figure 14). RNA:DNA was not significantly correlated to shell length, total brood, or reproductive output (Spearman Rank correlation, $p \geq 0.03$; data not shown).

Microbial analysis - Alpha diversity

Clam tissue had significantly lower diversity than water samples, for all of the alpha diversity metrics tested (Figure 15): Observed OTUs (H = 38.94, $p < 0.001$); Pielou's Evenness (H = 32.71, $p < 0.001$); Shannon's diversity index (H = 38.89, $p < 0.001$), and Faith's phylogenetic diversity (H = 38.22, $p < 0.001$).

Of the total 1,471 features found across the clam and water samples combined, 1,381 of the features were present only in the water samples, 29 were present only in the clam samples, and 61 were present in both. This overlap is visualized in Figure 16. Using all four measures of alpha diversity, water samples did not differ from one another across lake ($p \geq 0.12$). According to a Spearman Rank Order correlation, alpha diversity was not related to the numerical variables of clam shell length, total brood, or reproductive output ($p \geq 0.17$; data not shown).

Clam sample difference across lake depended upon the measure of alpha diversity used (Tables 7-10, Tables 15-16). Using Observed OTUs, Benewah L. clams were

indistinguishable from clams from any other population ($p \geq 0.07$). Bull Run L. clams were the same as Rose L. clams ($p = 0.65$) and Medicine L. clams were the same as Thompson L. clams ($p = 0.58$). All other pairwise comparisons were significant ($p \leq 0.04$).

Using Pielou's Evenness, Benewah L. clams similar to the other populations ($p \geq 0.2$) with the exception of clams from Medicine L. ($p = 0.02$). Clams from Bull Run L. were indistinguishable from the other populations ($p \geq 0.15$). Clams from Medicine L. also were similar to all other populations ($p \geq 0.47$) except Rose L. clams ($p = 0.01$). Clams from Rose L. and Thompson L. were not different ($p = 0.25$).

Using Shannon's diversity index, there were no differences across clams from any of the lakes ($p \geq 0.07$).

This pattern is the opposite using Faith's Phylogenetic Diversity: clams from Benewah L. (Table 15) were significantly different than clams from all lakes ($p \leq 0.04$) with the exception of clams from Bull Run L. ($p = 0.42$). Bull Run L. clams were different from all other clams ($p \leq 0.03$) except for those from Rose L. ($p = 0.1$). Clams from Medicine L. were different from all other clams ($p = 0.01$) except clams from Thompson L. (0.27). Clams from Rose L. and Thompson L. were different ($p = 0.01$).

Alpha diversity (Tables 15, 16) was significantly different between the ten possible "sample type + site" categories (five lakes * two sample types, clam and lake water) for the following alpha diversity metrics: Observed OTUs ($H = 42.50$, $p < 0.001$); Pielou's Evenness ($H = 36.47$, $p < 0.001$), Shannon's diversity index ($H = 40.22$, $p < 0.001$); and Faith's phylogenetic diversity ($H = 44.30$, $p < 0.001$) (Table 7-10). Within these samples, trends were fairly consistent in that the water samples largely did not

differ from one another, the clam samples were significantly different from the water samples (Kruskal-Wallis ANOVA, $p < 0.001$), and the clam samples were significantly different from one another (Kruskal-Wallis ANOVA, $p < 0.001$).

Microbial analysis - Beta diversity

The clam-associated microbiome structure was significantly different than their surrounding environmental water bacterial communities (Figure 17) (weighted UniFrac, pseudo-F = 60.22, $p = 0.001$). Using the "sample type + site" categorization, clam tissue and water samples had significantly different microbial community structures in the four metrics of beta diversity: Bray-Curtis distance (pseudo-F = 5.93, $p < 0.001$), Jaccard distance (pseudo-F = 4.27, $p < 0.001$), unweighted Uni-Frac (pseudo-F = 6.89, $p < 0.001$), and weighted Uni-Frac (pseudo-F = 10.86, $p < 0.001$). The patterns observed for weighted UniFrac were also consistent for the other beta diversity metrics analyzed, although the weighted UniFrac was the most conservative measure and produced the fewest significant pairwise comparisons (Table 11-14).

Using the weighted UniFrac (Table 13), the Rose L. and Bull Run L. clams were significantly different from all other clam populations ($p \leq 0.008$), but clams from Thompson L., Medicine L., and Benewah L. were indistinguishable from one another ($p \geq 0.109$). All of the clam-clam pairwise measures were significantly different using the other three measures of beta diversity (Tables 11, 12, 14).

The major taxa found in the clam samples were members of the phyla Bacteroidota, Firmicutes, and Spirochaeota, with mean percent relative abundances of 55.2, 17.9, 14.7, respectively (Figure 19). The major taxa found in the water samples were members of the phyla Actinobacteria, Proteobacteria, and Verrucomicrobiota with

mean percent relative abundances of 36.3, 23.6, and 17.5, respectively (Figure 19). Nineteen of the total 30 identified phyla were not present in the clam tissue samples; all 30 phyla were represented across the water samples. The five main genera in the water samples were *Sporichthyaceae, Terrimicrobiaceae, Bacteroidales*, *Pseudarcicella*, and *Chitinophagaceae* (Figure 21). The five main genera in the clam samples were *Bacteroidales, Chitinophagaceae, Mycoplasma, Alphaproteobacteria*, and *Clostrodium sensu stricto* 1 (Figure 20).

DISCUSSION

In this study I tested the hypotheses that exposure to trace metals requires metabolic investments that alter fingernail clam life history and that fingernail clams display a different microbial community than that of their environment, indicating a process of selection of microbial partners. In general, I found that clams from Benewah L., the reference site, displayed larger investment in both somatic maintenance and reproductive effort than did clams from the metal-impacted chain lakes. Among the four chain lake populations, clams from Bull Run L. had more investment in somatic growth while clams from Rose L. had a smaller investment in reproduction relative to somatic growth. Clams from Medicine L. and Thompson L. had the highest tissue Cd burden; across the five populations, tissue Cd was negatively related to shell length. I also found that the water microbial community was substantially more diverse than the clam microbiomes, and generally did not differ across the five lakes despite the limnological variation reported here and in previous work. In contrast, the five clam microbial communities were different both from one another and from their respective lake environment communities.

Lake water results

Due to sampling restrictions, I collected only one set of limnological measurements and therefore did not perform statistical analyses on them; I compare my results to those of Higbee (2017) and to published values, where available. Higbee (2017) conducted six monthly collections during the summer of 2016. She found that Benewah L. tended to have higher alkalinity, pH, and DO than did the chain lakes; I found that Benewah L. had the highest alkalinity and second-highest DO, and but an intermediate pH. In general, my hardness measurements were higher and my alkalinity measurements were lower than those collected during the 2016 field season. The day that we collected the samples, water temperatures were within 1-3°C of the 2016 August measurements. My DO and hardness measurements for Bull Run L. were slightly higher than those collected during the 2016 field season, but my pH and alkalinity measurements were lower. My overall measurements of Bull Run L. are consistent with the 2016 measurements, in that Bull Run L. has a lower pH and substantially DO content when compared to the other chain lakes.

Although I was only able to sample the lake water a single time, previous work in the drainage provides an overview for expected metal levels in the lake water and supports the consideration of these lakes as reference (Benewah L.), metal-impacted but relatively low metal content (Rose L.) and metal-impacted (Medicine and Thompson L.). The classification of Bull Run L. is unclear, as total lake water metal content is comparable to Medicine L., and the (on average) lower water hardness in Bull Run L. should result in higher metal bioavailability. As Higbee did not collect amphipods from Bull Run L., we cannot evaluate the classification of this lake within the context of lake

water metal levels and tissue metal burden (as discussed below). Higbee (2017) collected large volumes of lake water and boiled the samples to concentrate the metals prior to analysis by ICP-OES. Like Higbee (2017), I also acidified the samples prior to filtering them, thus providing an estimate of total metal, not just soluble metal (but I did not boil samples). Higbee found that water samples from Benewah L. and Rose L. had significantly lower levels of Cd and Pb than did Medicine L. Additionally, she found that Rose L. water had significantly lower As, Pb and Zn levels than did Medicine and Thompson L., reflecting the effect of the dam that limited the input of CDA River water and sediments into Rose L. The results reported here follow the same general trend. My single estimates of metals were within the seasonal ranges reported by Higbee for As and Cd in Medicine L., Pb in Bull Run L., and Zn within Rose, Bull Run, and Benewah L. My estimates of Pb and Zn in Medicine L. water were higher than what she reported; my estimates of Pb and Zn for Thompson L. were just below her seasonal ranges.

Comparable data are more often reported for the CDA River than for the lakes in its floodplain, as in reports from the USGS and as reviewed in Chapter 4 of the report prepared for an interagency and Tribal collaboration, the Restoration Partnership (LeJeune et al., 2000). For example, a USGS (2003b) report provided total metal levels for river water at the Cataldo site (just within the Superfund Site box noted in Figure 1 and the closest upstream site to the chain lakes), the CDA River near Harrison, which is just downstream from Thompson L., and the St. Joe River, upstream from where it enters Benewah L. The total metal levels described within Higbee (2017) and reported here are within the ranges reported for Cd, Pb and Zn at the river sites from 2009-2013, with the exception of our Rose L. values, which are lower.

Comparing our lake metal (As, Cd, Pb, Zn) results (those that were not below the limit of detection) with the EPA's guidelines for acute (CMC) and chronic (CCC) toxicity concentrations of metals in fresh water (EPA): Medicine L. exceeds the CMC and CCC concentrations for Cd, Pb, and Zn; Rose L. is below the CMC and CCC concentrations for Zn; Bull Run L. exceeds the CMC and CCC concentrations for Zn and Pb; and Thompson L. exceeds the CCC concentration for Pb. The CMC and CCC concentrations compared against here were adjusted for our lake water sample hardness (USEPA 2004). Comparing our lake water results to the Coeur d'Alene Tribal Water Quality Standards for CMC and CCC toxicity concentrations (LeJeune et al., 2000), Medicine L. exceeds both concentrations for all three metals. Bull Run L. Pb concentrations are higher than both the CMC and CCC, and Thompson L. has Pb concentrations higher than the chronic and nearing the acute concentrations.

Within the context of these water quality guidelines, and given previous work on the lake sediments (Sprenke et al. 2000), nearby river sediments (Farag et al. 1998) and surface waters (LeJeune et al. 2000, Mebane 2003b), it is reasonable to consider clams from the chain lakes to experience higher trace metal content than clams from Benewah L. My interpretations of clam growth and reproduction trends and the microbiome results are framed within this categorical consideration of sites rather than against metal presence *per se* as I do not have sufficient lake water data (i.e., monthly samples) to discriminate further among chain lakes nor to evaluate the other results within the context of metal exposure as a continuous variable.

Clam shell length and brood size

I predicted that clams from lakes with higher levels of metal pollution would exhibit higher somatic growth (shell length) relative to their reproductive output than would clams from the reference lake or from lakes with historically lower metal concentrations (Rose L.). This hypothesis is consistent with the predictions of the energy budget model (Sokolova et al. 2012), given the assumption that persisting in metal-polluted lakes creates metabolic demands that are not present for populations in sites that are not impacted by pollution.

Bivalves, like other aquatic organisms, will produce protective proteins when exposed to xenobiotics or other anthropogenic factors on both acute and chronic time frames (Perceval et al. 2004, Ivanina et al. 2011, Baldi et al. 2013, Wang et al. 2014, Lokmer and Mathias Wegner 2015); depending upon the type of response needed, this production can come at the cost of other physiological processes. For example, a population of *Corbicula fluminea* downstream from a power plant exhibited elevated production of heat shock proteins; this was accompanied by a downregulation in several energy metabolism enzymes and proteins protective against oxidative damage (Falfushynska et al. 2016). Some proteins protect against damage from trace metals by binding to the ions, both the excess essential metal ions and any nonessential metal ions present in the tissues (Marie et al. 2006). In addition to metal-sequestering proteins, any oxidative damage that results from excess metal ion presence requires the production of antioxidants (to detoxify the free radicals) and/or repair proteins, which also contribute to the cost of somatic maintenance in metal-exposed animals, reducing available energy for other processes (e.g., Ivanina and Sokolova 2008). The increased energetic demands of these protective factors are predicted to come at the cost of somatic growth and

reproductive output (Ivanina and Sokolova 2008, Sokolova et al. 2012). Future work on fingernail clams in the Coeur d'Alene river drainage should include an evaluation of protein expression within the context of shell length, reproductive output, and tissue metal burden to evaluate whether the differences noted in growth and reproduction here reflect metabolic investment in cellular-level protection.

One factor limiting our ability to determine whether clams in the metal-impacted chain lakes exhibited evidence of the (putative) costs of metal tolerance by comparing chain lake populations with clams from Benewah L. and with populations from other, unpolluted habitats, is our uncertainty over which species are present. There are four genera of fingernail clams (*Corbicula*, *Sphaerium*, *Musculium*, and *Pisidium*); across most habitat types, species from multiple genera will coexist (Mackie 2007), with one unpolluted site in Ottawa containing 15 species from three genera (Kilgour 1988). Identification of fingernail clams to species requires examination of the angles of the shell hinges and other finely detailed morphological traits, for which we lack the appropriate microscopes. While we cannot confirm the species collected in August 2020, we previously collaborated with Taehwan Lee (Museum of Zoology, University of Michigan). Dr. Lee has constructed a phylogeny of fingernail clams using nuclear and ribosomal gene sequences (Lee and Foighil 2003). Dr. Lee identified two *Musculium* species (*M. lacustre* and *M. securis*) and a single individual of either *P. variabile* or *P. casertanum*, in a 2012 collection from Benewah L. Given his uncertainty over the *Pisidium* species, his warnings (pers. comm.) about identifying immature clams, and our inability to discriminate among these species, I will compare my results to previous work on *Musculium* spp.

Environmental stress has been linked to changes in somatic growth and/or reproductive activity in in *Musculium* spp. For example, shell length and metabolic markers, including glycogen content, in *M. transversum* were negatively affected by acute (56 day) decreases in diet quality (Naimo et al. 1998). Natality in *M. securis* responded either directly or inversely to changes in pollutant concentrations (Mackie 1978a), depending on pollutant type (none were metals). I found that clams from the reference lake (Benewah L.) had significantly longer shells, indicating a higher somatic growth measurement and/or longer lifespan than did clams from any of the four chain lakes. I also found that clams from Bull Run L. had significantly longer shells than did clams from the other three chain lakes. I also found a potential negative relationship between tissue Cd burden and shell length, although it is affected by the inclusion of Benewah L. clams which displayed long shell lengths and low tissue Cd concentrations. In the absence of a full season of clam size/frequency data (as in, McKee 1981, Joyner-Matos et al. 2011) and confirmation of species, these data should be interpreted cautiously as the populations may have been at different stages of an annual cycle of growth. Nonetheless, the difference between clams from Benewah L. and the chain lakes partially supports my hypothesis and is in agreement with the energy budget model view of somatic growth as a category that is sacrificed in clams that are tolerating trace metal (Cd) stress (Sokolova et al. 2012). The season-long size/frequency study of amphipods in these lakes by Higbee (2017) indicated that month, and the interaction between lake identity and month, were the only significant factors explaining variation in amphipod length. In general, amphipods from Benewah L. and from Medicine L. tended to be longer than those from other lakes, indicating both the influence of differential timing of

reproductive events (evident in her size/frequency distributions) and potential impacts of metal exposure on growth rates. Finally, I found that the Bull Run L. clams had intermediate shell lengths, the first of several findings of intermediate performance in this population, which is discussed further below.

Across several metrics, the clams from Benewah L. exhibited higher investment in reproduction. All clams collected from Benewah L. were brooding and nearly half of them contained offspring at multiple developmental stages. Clams from Benewah L. had the highest reproductive output (total brood divided by shell length), and the highest maximum brood size (which likely reflects their overall longer shell lengths). These results are in agreement with my hypothesis and with the energy budget model (Sokolova et al. 2012). *Musculium* spp. reproductive traits are variable across and within population and are responsive to acute changes in environmental conditions (e.g., Mackie 1976). In general, the proportion of clams that are brooding and the proportion that contain multiple developmental stages (in those species that do simultaneous litters) are considered indicators of populations in optimal conditions. By this logic, clams from Rose L. were in the least optimal conditions, as nearly half of the clams were not brooding, and only 4% had broods at multiple stages. This is confirmed by the reproductive output calculation, for which clams from Bull Run L., Medicine L., and Thompson L. all displayed higher reproductive output than did clams from Rose L.

Finally, across a wide range of studies of sphaeriids (for review, Mackie 2007), shell length tends to be a significant predictor of total brood size, regardless of habitat type or other environmental factors (e.g., water temperature). I therefore examined the relationship between total brood count and shell length for all clams that had visible

brood. In four of the five populations I found the expected, positive relationship between adult shell length and brood size (this was not reported for Medicine L. as the data did not meet assumptions of regression, but the relationship did appear to be present). This relationship was strongest for clams from Benewah L., but as the slopes of the regression lines did not differ significantly across the clam populations, we cannot use this metric to discriminate amongst the populations in relative investment in reproductive output versus somatic growth. I note that the regression for Benewah L. clams was impacted by one clam that was quite distinct from the rest (shell length of 11 mm, total brood of 7). All offspring in this large clam were shelled juveniles that were held in the mantle cavity; this is the developmental stage immediately prior to juvenile extrusion. It is possible that, prior to collection, this clam extruded another set of larvae and the brood count of 7 is an underestimate of this clam's fecundity. Although I did not test whether this clam would be identified as an outlier and did not report regression results with this clam omitted, it is clear from a visual inspect of the results that if this clam were omitted, the relationship between total brood and shell length in Benewah L. clams would be considerably stronger and more distinct from the other populations.

Clam tissue metals

We expected the chain lake clam populations to have higher levels of metals based on previous work in the chain lakes (Schoonover 2013, Higbee 2017) and the fact that bivalves are biomagnifiers (Luoma 1983) and previous work confirms trace metal presence in the lake water and sediment. Trace metals disruption of cellular function (Ballatori 2002) occurs at particularly high rates along respiratory epithelia in aquatic organisms, where tissue surface area and water flow rates are high. In *C. fluminea,* tissue

metal content reflects the patterns of the metal content of the sediments in their environment (Luoma 1983). Exposure to metal through food seems to result in greater metal accumulation in the muscle and digestive glands specifically, while whole body metal contamination is affected by surface absorption and can mask internal tissue metal accumulation (Luoma 1983). *Sphaerium* spp. body tissue reflects concentrations of Cu, Cd, and Pb representative of the sediment metal concentrations, regardless of the surrounding water metal content and *Sphaerium* spp. body tissue concentrates Zn in significantly higher concentrations than is found in neighboring sediments (Anderson 1977). Finally, *Musculium* spp. collected from an unimpacted site in eastern WA that were exposed for 40+ days to sediment from Killarney L., typically considered to be the chain lake with the highest sediment metal content, accumulated Cd, Pb and Zn in a broadly dose-dependent manner, to a maximum of 4.5 μg Cd, 600 μg Pb, and 300 μg Zn/g (whole clam) tissue (Schoonover et al. 2016).

Clams from Medicine L. and Thompson L. had significantly higher levels of tissue Cd than did clams from Benewah L. The Medicine L. clams also had significantly higher tissue Cd levels than clams from Rose L. These findings are in accordance with Higbee (2017) who found the higher tissue Cd levels in amphipods from Medicine L. and Thompson L than in amphipods from Rose L. and Benewah L. Farag et al. (1998) also found significantly higher concentrations of Cd, Pb, and Zn in benthic macroorganisms sampled from the drainage region containing Medicine L. and Thompson L. Based on the data from Higbee (2017), in which Bull Run L. water had comparable total Cd content but lower hardness than did Medicine L. water, the Bull Run L. clams had lower than

expected tissue Cd (note that Higbee did not find amphipods in Bull Run L. and thus does not report tissue metal burden for animals from this lake).

the Bull Run L. clams had lower than expected tissue Cd. This may reflect other factors in the lake that would make the metals less bioavailable, such as high dissolved organic carbon, and/or compensation by the clams in response to chronic hypoxia. Our results are opposite to those of Tran et al (2001) who acutely exposed Corbicula to low DO and studied Cd uptake parameters. In this case, with acute hypoxia exposure, ventilation rates and Cd accumulation increased. The applicability of this study is somewhat limited as the Corbicula were acutely exposed to hypoxia and in Bull Run L. the hypoxia, as far as we can tell, is more chronic than acute. In populations that are consistently exposed to hypoxia, such as the marine clam *Ruditapes decussatus* (Sobral and Widdows 1997), clams tend to decrease their activity levels when exposed to hypoxia, decreasing both ventilation rates and clearance (water filtration) rates, and can maintain these responses for a considerable time while meeting the reduced metabolic needs with aerobic metabolism. Comparable responses have been documented in freshwater fingernail clams (for review, Mackie 2007). If future, season-long studies confirmed a pattern of low tissue Cd in Bull Run L. clams, then those results would need to be interpreted within the context of lake water dissolved organic compound levels and/or metrics of clam metabolism, such as balance between aerobic and anaerobic metabolism.

Due to sampling restrictions, we were only able to obtain one set of lake water metal samples and therefore cannot assess whether lake water metals correlate with tissue metal burden. In the 2016 amphipod study (Higbee 2017), only tissue Pb burden was

correlated with lake water metal content, which was not unexpected (for review, Luoma 1983, Rainbow and Luoma 2011).

RNA:DNA

Nucleic acid ratios (e.g. RNA:DNA, RNA:protein) have been used as an estimation of short term growth in several organisms (Dahlhoff 2004, Norkko and Thrush 2006), and the RNA:DNA is a reliable indicator of stress response (Revankar and Shyama 2009) in many organisms, including bivalves. We hypothesized that the RNA:DNA would be lower, reflecting decreased overall investment in protein synthesis despite any tissue-specific synthesis of protective proteins (Lannig et al. 2006), in the tissues of clams from the metal-impacted lakes than in the tissue of clams from the reference lake. However, the RNA:DNA of the clams analyzed in this study were not significantly different between the lakes. This may reflect, in part, constraints of the small sample size (an n of 6 from each lake) and of having to pool all tissues within a clam, which eliminates my ability to report the tissue-specific patterns in RNA:DNA that typically are reported for larger bivalves.

With the exception of clams from Bull Run L., the clams in this analysis had RNA:DNA similar to those of fingernail clams sampled along an O_2 gradient (with a comparably small sample size), although the ratios in that study differed significantly between habitats (Joyner-Matos et al. 2007). The RNA:DNA reported here are more similar to those seen in starved *Ruditapes decussatus*, a marine bivalve, although this pattern was measured over time rather than using a one-time sampling event (Chicharo 1995). It is possible that my reliance upon measuring the nucleic acids in samples that were prepared using a kit that is optimized for DNA extraction resulted in artificially low

RNA yield (which would be consistent across samples but which may have masked some variance). As it is not feasible to conduct tissue-specific work in these small clams, future characterizations of RNA:DNA should be from samples that are processed through more traditional extraction techniques (e.g., Trizol Reagent) that isolate RNA and DNA with comparable efficiency.

Comparisons among lake water and clam tissue microbial communities

I hypothesized that the clam bacterial community would be different from the bacterial community in their corresponding water samples (Walke et al. 2014, Kruger 2020). This was in part based on the presence of metal-reducing bacteria inside Manila clam tissues and the presence of bacterial xenobiotic degradation transcripts within Manila clam hepatopancreas in prior studies (Baldi et al. 2013, Milan et al. 2018). Metal-resistant bacteria, bacteria with plasmid-borne metal resistance operons, are often found in metallurgic wastes (Mergeay et al. 2003). Based on the metrics of alpha and beta diversity that I evaluated, the clam microbial communities were significantly different from the communities represented in their lakes, which suggests the possibility of a selection mechanism (Walke et al. 2014). Because the analyses I described here did not include identification of bacterial function, I cannot assess whether fingernail clams may be forming relationships that facilitate their tolerance of metal mixtures, such as those with metal-reducing bacteria.

According to our metrics of alpha diversity, there were no differences between the water samples from the five lakes. This is interesting given that the lakes are located along a gradient of trace metal pollution and differ in some limnological features (Higbee 2017), and that previous work in Rose L. identified the presence of a fungus that

appeared to be colonizing amphipod tissues (Higbee 2017). While the presence of pollution has been associated with altered wetland and lake microbial communities (Newton and McLellan 2015, Aguinaga et al. 2018, Liao et al. 2019), it is possible that we were not able to capture differences among lakes because we only sampled once (Higbee 2017 reported a strong seasonal impact on nearly every lake water variable). The similarity among the chain lakes likely reflects, in part, that they are in the same drainage and open for recreation (Kosek et al. 2019). It is less clear why Benewah L. is indistinguishable from the chain lakes.

According to our measures of beta diversity, only the pairwise comparison of lake water samples that was significant was Benewah L. and Thompson L.; this pair was significant using the Bray-Curtis (reports differences in presence/absence and abundance of features) and Jaccard (presence/absence of features) metrics. It is not immediately apparent why this pair of water samples would be significant and others would not. Thompson L. water has higher total metal content (and slightly lower alkalinity) and the clams and amphipods living in Thompson L. have higher tissue metals than do those in Benewah L., (results reported here and in Higbee 2017). However, as these are features shared by other chain lakes, we cannot at this time explain why this pairing is significant for these two measures of beta diversity.

A large percentage of the taxa present in the lake water samples were labeled as uncultured or as novel species with very little physiological information available. In addition to these identified unknowns, the most common identifiable taxa included members of Actinobacteria, Bacteroidetes, Verrucomicrobia, Firmicutes, and Gammaproteobacteria, which overlap with those reported in other freshwater lake and

wetland studies (e.g., Newton et al. 2006, Newton et al. 2011, Bodelier and Dedysh 2013, Lv et al. 2014, Zhang et al. 2018).

Pairwise clam tissue alpha diversity comparisons varied with the alpha diversity metric used, and it is not possible to draw one overall trend from the Observed OTUs, Pielou's Evenness, or Shannon's diversity results (Tables 7-10, Tables 15-16, Figure 15). As the microbiome of the fingernail clam has not been characterized, I cannot compare this variability to species-specific published work, and as alpha diversity is easily affected by experimental measurement errors (Willis 2019), it is possible that this variation is an artifact of my low sample size, sequencing depth, and/or methodological issues sampling the whole clam microbial community. However, two recent studies of microbial taxa associated with zebra mussels reported similar numbers of observed OTUs (Mathai et al. 2021). Similarly to our fingernail clam samples, both their OTU richness and Shannon's diversity indices were lower in the zebra mussels than in their water (Mathai et al. 2020). Faith's Phylogenetic Diversity indicated that the fingernail clam populations were significantly different from one another overall (Table 9) meaning that despite the variability of richness and evenness between populations, the populations differ in their phylogenetic diversity. Namely, clams from Benewah L. were significantly phylogenetically diverse (distinct from) when compared to clams from all other lakes besides Bull Run L. Clams from Bull Run L. were significantly phylogenetically diverse from clams from all other lake except Rose L., and clams from Medicine L were significantly phylogenetically diverse from all other clams except Thompson L.

According to our measures of beta diversity, the clam tissue samples had different microbial communities across the five lakes, despite the finding that the five lake water

communities were indistinguishable. The only exception to this was seen when using weighted Uni-Frac distance, a measure based on the presence and absence and abundance of sequences within the context of a phylogenetic tree (Lozupone et al. 2007). In this pairwise comparison, Benewah L. clams did not have a different microbial community structure than clams from Medicine L. or Thompson L (Table 13). This is interesting because, as mentioned previously, clams from both Medicine L. and Thompson L. had significantly higher tissue Cd levels than clams from the reference lake (Benewah L.) (Figure 12). Across all measures of beta diversity, Rose L. clams were different from all other clams, which is consistent with anecdotal observations made both during clam handling for this project and from Chantilly Higbee. The Bull Run L. clams were also different from all other clams across all four measures of beta diversity, which may reflect the multiple known features in the lake (metals, slight acidity, considerable hypoxia) in addition to as yet unknown features.

Across clams from all five lakes, I found the same five identifiable phyla present (the sixth common phyla was "unidentified bacteria"): Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetota, and Verrucomicrobia (Figure 20, 21). These phyla are represented in other freshwater lake and wetland microbial studies (Aguinaga et al. 2018). Benewah L. and Medicine L. clams did not contain any additional phyla, but clams from the other three lakes contained members of Actinobacteriota. Additionally, Bull Run L. clams contained Bdellovibrionota and Chloroflexi; Rose L. contained Chloroflexi, Cyanobacteria, and Planctomycetota. A paper on zebra mussel microbial communities also identified members of Proteobacteria and Bacteroidetes (Mathai et al. 2020).

Actinobacteriota are large group of gram-negative, ubiquitous bacteria with extensive metabolic flexibility that form a diverse range of associations with other organisms (Barka et al. 2016). Bacteroidetes (gram-negative) and Firmicutes (gram-positive) are associated with gut microbiota; whether this representation in the clam tissue samples is from selection of a gut microbiome or from fecal contamination of the lake water is unclear (Ibekwe et al. 2003, Boehm and Sassoubre 2014, McLellan and Eren 2014). Members of Bdellovibrionota are gram-negative obligate predators of other gram-negative bacteria (Iebba et al. 2014, Bratanis et al. 2020). Verrucomicrobia is a group of gram-negative bacteria associated with both mucin degradation in the gut microbiome and with soil and water microbial communities (Lee et al. 2009).

Members of Spirochaetota are gram-negative motile bacteria, known for their distinctive corkscrew shape, and can exist in a range of habits including soil, arthropod guts, and vertebrates, where they are often pathogenic (Gupta et al. 2013). Groups in the phylum Chloroflexi are known for their filamentous morphology or ability to photosynthesize (Hanada 2014, Speirs et al. 2019). Planctomycetes are gram-negative bacteria associated with soil and water across the globe and contain many members involved in carbon and nitrogen cycles and the anerobic oxidation of ammonia (Wiegand et al. 2018). Cyanobacteria (gram-negative) are an ancient group of photosynthetic bacteria, capable of nitrogen fixation and the degradation of otherwise toxic compounds (e.g. those related to pesticides) (Singh et al. 2016).

When comparing the features present in the lake water and in the clam samples, I found only 61 features (4.1% out of the 1, 471 total features) in common between the clams and their lake habitats (Figure 16) and was able to positively identify four to the

order, family, or genus. This relatively low number of common organismal-environment features is the opposite trend to that found with amphibian skin microbiomes and their environment (Walke et al. 2014). Similarly to the features present in the lake water samples, the majority of those found both in the water and clams are uncultured or novel species. Those taxa that are broadly identifiable include members of the order Bacteriodales, which contains genera commonly associated with human gut microbiota and/or fecal contamination present in an environment (Boehm and Sassoubre 2014, McLellan and Eren 2014, Coyne and Comstock 2019).

Features associated with the family Chitinophagaceae were also present in both sample types. Chitinophagaceae is a relatively newly-reclassified family (Kämpfer et al. 2011) of anaerobic bacteria that contains a variety of taxa associated with soils including *Sediminibacterium*, *Flavisolibacter*, and *Terrimonas* (Kämpfer et al. 2011). Chitinophagaceae has been associated with marine clam biodeposits (lumps of organic matter) (Murphy et al. 2019).

Other identifiable features present in both samples included features associated with the genus *Mycoplasma* and the genus *Clostridium* (*sensu stricto*). *Mycoplasma* spp. are readily found in nature and have been associated with wetland communities (Ibekwe et al. 2003), but are largely known as pathogens and parasites to plants and animals, including humans (Razin and Hayflick 2010). Members of the genus *Clostridium* are obligate anaerobes and although some are pathogenic, others play a key role in gut microbial communities due to their production of butyrate (a short chain fatty acid that regulates several components of intestinal heath) (Canani et al. 2011, Lopetuso et al. 2013).

Originally, I had intended to evaluate the clam associated microbes within the context of lake water and clam tissue trace metal levels, to evaluate whether there is evidence that the clams are selecting for microbes that may assist with tolerance of this environmental stressor. This finding would be consistent both with the amphibian studies (McKenzie et al. 2012, Walke et al. 2014, Kruger 2020) and those with marine bivalves (Baldi et al. 2013, Milan et al. 2018, Lim et al. 2019, Murphy et al. 2019). However, given the small number of features in the clam microbiome, and the high proportion that are currently unidentified, I cannot, at this time, evaluate this hypothesis. It is possible that full evaluation of this will require will require microbiome characterizations in other populations of fingernail clams, to increase the number of identified microbes, and/or utilization of curated databases of bacteria to identify the sequences.

Microbiome methodological limitations

Across all five lakes, the microbial communities of the clam samples were significantly less diverse than the microbial communities of their environmental lake waters. As this is, to my knowledge, the first description of a fingernail clam microbiome, we cannot evaluate whether the clam microbiome diversity is unusual. The low sequence (identifiable sequence of base pairs) count and low microbial diversity (based on identifiable sequence segments) seen in the clam samples may represent a mechanism for microbial selection (e.g., McKenzie et al. 2012, Walke et al. 2014, Kruger 2020) or a methodological error specific to the clam sample processing. Because I completed the optimization of my microbial methods prior to finding a zebra mussel paper (Mathai et al. 2021), my methodology was developed based on vertebrate and insect microbiome sampling (Walke et al. 2014). After I had developed my methods, I found a study of

zebra mussels (*Dreissena polymorpha*) (Mathai et al. 2020); as these mussels tend to be slightly larger than the fingernail clams that I dissected, their methods are only partially applicable. Mathai et al. (2020) cleaned the shells with 70% ethanol, handled the internal tissues differently than I did, and extracted DNA using the DNeasy PowerSoil Kit rather than the Blood and Tissue kit (Mathai et al. 2020). It is not clear whether Mathai et al. analyzed mixed or individual tissues so I cannot compare our methods in that aspect. Similarly to my fingernail clams species, the zebra mussel bacterial community was dominated by Proteobacteria, Actinobacteria, Cyanobacteria, Bacteriodetes, and Planctomycetes. It is possible that fingernail clam tissue requires a different dissection and DNA extraction protocol in order to capture the full microbial community associated with their tissues. Additionally, I wanted to avoid identifying microbes that were present on the outside of the clam shell, as these might or might not be deliberately "cultured" epibionts, which cannot be evaluated before the microbiome of the clam tissues are known. Taking steps to minimize shell-microbe contamination is consistent with a recent study of the microbiome of zebra mussels (Mathai et al. 2021). Given the fragility of the fingernail clam shells, my shell-removal process may have inadvertently decreased the microbial population within the mantle cavity fluid and/or on the surface of tissues such as the gill.

The finding of low clam sequence counts may be further complicated by the loss of 43 sequences from both water and clam tissue samples; these sequences were identified from contamination in the DNA extraction controls and therefore may have contaminated the samples. Although roughly half of these sequences were only present in the water samples, it is possible this removal changed the results of the clam microbiome

analysis. The contamination of the DNA extraction controls was irregular and seemingly unexplainable, fitting with literature on DNA extraction kit contamination, for which low diversity samples are particularly vulnerable (Paniagua Voirol et al. 2020). For further reference, see recent work on the "kitome" and "splashome" (Mohammadi et al. 2005, Glassing et al. 2016, Stinson et al. 2019, Olomu et al. 2020, Paniagua Voirol et al. 2020).

Conclusions and future studies

Here I explored whether I could detect evidence that the clams in the metal-polluted lakes exhibit trade-offs in growth and/or reproduction that we predict to occur in animals that tolerate anthropogenic stressors. I also explored whether those clams were forming relationships with microbes that may, through altering energetic reserves available to the clams facilitate heightened tolerance of the metal pollution.

While I found the overall expected patterns with respect to clam shell length and reproduction, that clams from the unimpacted site have higher fitness than those from metal-polluted lakes, I was not able to discriminate clearly among the four chain lakes. This was somewhat surprising, given differences in limnology between Rose L. and the other chain lakes (lower hardness but higher alkalinity, and fewer metals in Rose L., Higbee 2017) and between Bull Run L. and the other chain lakes (hypoxic and acidic water in Bull Run L.). The clam population in Bull Run L., in particular, merits additional attention, as the potential consequences of the limnology and metal content of this water body could not be evaluated in Higbee 2017 because no amphipods were found. It is possible, consistent with previous work with hypoxia-exposed sphaeriids (Joyner-Matos et al. 2007), that the clams in Bull Run L. have the highest fitness of any chain lake population because they are hypoxic. The results from my single collection event tend to

support that interpretation, as Bull Run L. clams were the largest, most reproductively active, and were the easiest to locate of any of the chain lake populations. They also had lower tissue Cd, although that result, if confirmed in future studies, would need to be evaluated within the context of season-long limnology and clam bioenergetics. Future studies of the chain lake clam populations should be conducted monthly during the summer season and should include an estimate of population size, to evaluate whether the differences in growth and reproduction that I report here scale up to population-level effects.

The microbial community of Benewah L. clams was comparably diverse and evenly spread to the clam communities of the other lakes, but was significantly less phylogenetically diverse. The Benewah L. clam microbial community was significantly different than the Benewah L. water community and from the clam communities of the other four lakes. Determining whether this represents the absence of metal pollution, or the limnological differences between Benewah L. and the chain lakes, requires other clam microbial communities to be characterized. Interestingly, the Rose L. clam microbial community was the only one containing members of the phyla Actinobacteriota, Chloroflexi, Cyanobacteria, and Planctomycetota in addition to the five identified phyla present in all clams samples. In Higbee (2017) and in the current work, animals collected from Rose L. are less likely to survive, tend to have fungal growth, and the water samples tend to be different from those of other lakes (different odor, color). Determining whether these factors impact the clam fitness and amphipod fitness (reported here and in Higbee 2017) requires full characterization of the microbial community in Rose L., but should be prioritized, as Rose L. supports active recreation, including fishing and a resort, and it is

possible that there are microbes in the water (or in the amphipods and clams, which are consumed by the fish) that may be harmful to humans. While all of the lakes used in this study are sites for recreation, among the chain lakes, more members of the general public have been observed during collection visits at Rose L. than at Bull Run L. or Thompson L., and usage of Medicine L. appears to be intermediate.

While I did not detect significant differences in RNA:DNA with six clams per lake, it is possible that an investigation of energy reserves, particularly glycogen content and/or metabolic enzyme activity levels, coupled with a season-long characterization of clam size/frequency distribution and reproductive patterns, would illustrate a relationship between measures of energetic partitioning, life history results, and trace metal exposure. Within this more complete framework it may be possible to tease apart relevant differences in the clam microbial communities that allow us to test whether the low diversity of clam microbial community, compared to that of the water, represents active selection on the part of the clam and/or selection by the microbes.

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TABLES

Table 1: DNA extraction batch details

DNA Batch	Sample ID	Dissection C.	Extraction C.	C pooled + seq
Clam 1	All lakes: clam # 01, 02 DC1 EC 1	Clean	Clean	Not necessary
Clam 2	All lakes: clam # 03, 04 DC2 EC 2	Clean	Clean	Not necessary
Clam 3	All lakes: clam # 05, 06 DC3 EC3	Clean	Contaminated	Yes
Clam 4	DC4 DC5 EC4	Clean	Contaminated	Yes
Water 1	T5, C5, R5 WEC1	N/A	Contaminated	Yes
Water 2	C1, M1, R1, R2, M2, B1, B2, T1, C2, T2, T3 WEC2	N/A	Contaminated	Yes
Water 3	R3, B3, M3, T4, C3, C4, B4, B5, M4, R4, M5 WEC3	N/A	Contaminated	Yes

DC#= Dissection control (#) EC = Extraction Control, WEC = Water Extraction Control
BR= Bull Run L., R= Rose L., T= Thompson L., M= Medicine L., C= Benewah L.

Table 2. Limnological features of the five lakes.

Lake	Temperature (°C)	DO (mg/L)	pH	Alkalinity ($CaCO_3$, mg/L)	Conductivity (µS/cm)	Hardness (mg/L)
Rose	21.3	7.5	7.6	4.8	36.4	13.82
Bull Run	17.6	3.1	5.4	2.2	21.6	13.11
Medicine	21.9	8.0	5.9	12.1	46.0	20.86
Thompson	24.2	8.3	6.3	15.7	50.3	24.55
Benewah	23.5	8.0	6.1	24.5	52.4	26.78

Data collected on August 7, 2020. Temperature, Dissolved O_2 (DO), pH and conductivity taken in triplicate measures and averaged to present a single measurement. Alkalinity measured via titration, one sample per lake. The hardness of one water sample per lake was calculated from Ca and Mg levels determine by ICP-OES; see Methods for calculation.

Table 3. Total metals in the five lake water samples.

Lake	As (mg/L)	Cd(mg/L)	Zn(mg/L)	Ca (mg/L)	Mg (mg/L)	Pb (mg/L)
Rose	<LOD	<LOD	0.016	3.22	1.40	<LOD
Bull Run	<LOD	<LOD	0.120	2.92	1.41	0.026
Medicine	0.017	0.0017	0.163	5.00	2.03	0.307
Thompson	<LOD	<LOD	0.015	5.95	2.35	0.007
Benewah	<LOD	<LOD	0.006	7.04	2.23	<LOD

Total metal content from a single water sample per lake was determined by ICP-OES. < LOD, below the limit of detection.

Table 4: LOD and LOQ values and list of samples below the limits.

		As (mg/L)	Cd (mg/L)	Zn (mg/L)
	Water LOD	0.002	0.00005	0.001
	Water LOQ	0.005	0.00015	0.003
	Tissue LOD	0.003	0.0001	0.000
	Tissue LOQ	0.010	0.0002	0.001
Water samples <LOD for each metal		Thompson L. Benewah L.	Rose L. Thompson L. Benewah L.	
Water samples <LOQ		Rose L. Bull Run L.	Bull Run L. Medicine L.	
Tissue samples <LOD		*all samples	DE01, DE05, DE10	
Tissue samples <LOQ		*all samples	DE26	

Wavelength for As1890, Cd2144, Na2138, Ca422, Mg2795, Pb2169. DE, sample ID abbreviation.

Table 5. Shell length and reproductive data of the five clam populations.

	Rose L.	Bull Run L.	Medicine L.	Thompson L.	Benewah L.
Sample Size	24	57	33	32	42
Shell length (mm)[a]	3.77 (0.6) [2.5 – 5]	4.89 (0.5) 3.8 - 6.1	4.22 (0.8) 3 - 7	4.03 (0.9) 2.9 - 8	6.52 (1.4) 4.9 - 11
% without brood	45	3	9	15	0
Average brood[a]	1.25 (1.5) [0 – 5]	4.79 (3.2) [0 – 16]	3.7 (3.5) [0 – 17]	3.25 (2.7) [0 – 12]	9.8 (5.6) [3 – 29]
% with multiple stages present	4	21	21	16	43
Reproductive output (Brood/shell length)[ab]	0.33 (0.39) [0 – 1.25]	0.97 (0.61) [0– 2.91]	0.83 (0.68) [0 – 3.4]	0.80 (0.61) [0 – 2.31]	1.48 (0.64) [0.53 – 3.1]

[a]Data presented as mean (SD) and range [min – max].
[b]Summary calculations included clams that were not brooding.

Table 6. Clam tissue metal content.

Lake	Sample ID	Cd (µg/g)	Zn (mg/g)*
Rose L.	DE05 (R07)	0.18	0.18
Rose L.	DE10 (R08)	0.07	0.43
Rose L.	DE12 (R09)	0.28	2.75
Bull Run L.	DE11 (B11)	0.59	0.22
Bull Run L.	DE04 (B12)	0.33	0.29
Bull Run L.	DE18 (B13)	0.46	
Bull Run L.	DE15 (B14)	0.72	
Bull Run L.	DE20 (B15)	0.35	
Medicine L.	DE02 (M07)	3.35	1.12
Medicine L.	DE07 (M08)	2.52	
Medicine L.	DE13 (M09)	3.17	
Medicine L.	DE17 (M10)	5.32	
Medicine L.	DE21 (M11)	3.03	
Thompson L.	DE03 (T11)	2.17	
Thompson L.	DE08 (T12)	2.70	2.08
Thompson L.	DE14 (T13)	1.68	0.28
Thompson L.	DE25 (T14)	1.53	
Benewah L.	DE09 (C11)	0.28	0.13
Benewah L.	DE01 (C12)	0.01	
Benewah L.	DE19 (C13)	0.12	
Benewah L.	DE22 (C14)	0.12	0.11
Benewah L.	DE23 (C15)	0.16	0.60
Benewah L.	DE26 (C16)	0.06	0.35
Benewah L.	DE27 (C17)	0.15	0.11

*Empty cells represent samples that were omitted due to high Zn in method blank.

Table 7: Pairwise comparisons for Observed Features/OTUs (alpha diversity)

Group 1	Group 2	H	p-value*
Clam Benewah L	Clam Bull Run L.	0.52	0.47
	Clam Medicine L	3.2	0.07
	Clam Rose L	0.21	0.64
	Clam Thompson L	3.04	0.08
	Water Benewah L	7.57	**0.01**
Clam Bull Run L.	Clam Medicine L	5.83	**0.02**
	Clam Rose L	0.21	0.65
	Clam Thompson L	4.11	**0.04**
	Water Bull Run L	7.53	**0.01**
Clam Medicine L	Clam Rose L	4.91	**0.03**
	Clam Thompson L	0.31	0.58
	Water Medicine L	7.57	**0.01**
Clam Rose L	Clam Thompson L	4.81	**0.03**
	Water Rose L	6.82	**0.01**
Clam Thompson L	Water Thompson L	6.82	**0.01**
Water Benewah L	Water Bull Run L	1.33	0.25
	Water Medicine L	0.27	0.6
	Water Rose L	0.01	0.92
	Water Thompson L	0.89	0.35
Water Bull Run L	Water Medicine L	0.4	0.53
	Water Rose L	0.53	0.46
	Water Thompson L	0.01	0.92
Water Medicine L	Water Rose L	0.1	0.75
	Water Thompson L	0.1	0.75
	Water Thompson L	0.88	0.35
Water Rose L	Clam Bull Run L.	1.33	0.25

*Groups that differed significantly from one another are bolded.

Table 8: Pairwise comparisons for Pielou's Evenness (alpha diversity)

Group 1	Group 2	H	p-value*
Clam Benewah L	Clam Bull Run L.	0.1	0.75
	Clam Medicine L	5.03	**0.02**
	Clam Rose L	1.63	0.2
	Clam Thompson L	0.03	0.86
	Water Benewah L	7.5	**0.01**
Clam Bull Run L.	Clam Medicine L	2.08	0.15
	Clam Rose L	1.2	0.27
	Clam Thompson L	0.13	0.72
	Water Bull Run L	5.63	**0.02**
Clam Medicine L	Clam Rose L	7.5	**0.01**
	Clam Thompson L	0.53	0.47
	Water Medicine L	4.03	**0.04**
Clam Rose L	Clam Thompson L	1.32	0.25
	Water Rose L	6.82	**0.01**
Clam Thompson L	Water Thompson L	3.15	0.08
Water Benewah L	Water Bull Run L	0.88	0.35
	Water Medicine L	0.53	0.46
	Water Rose L	0.1	0.75
	Water Thompson L	0.53	0.46
Water Bull Run L	Water Medicine L	0.01	0.92
	Water Rose L	1.84	0.17
	Water Thompson L	0.01	0.92
Water Medicine L	Water Rose L	0.53	0.46
	Water Thompson L	0.01	0.92
	Water Thompson L	0.53	0.46
Water Rose L	Clam Bull Run L.	0.88	0.35

*Groups that differed significantly from one another are bolded.

Table 9: Pairwise comparisons for Faith's Phylogenetic Diversity (alpha diversity)

Group 1	Group 2	H	p-value*
Clam Benewah L	Clam Bull Run L.	0.64	0.42
	Clam Medicine L	5.77	**0.02**
	Clam Rose L	4.03	**0.04**
	Clam Thompson L	4.03	**0.04**
	Water Benewah L	7.5	**0.01**
Clam Bull Run L.	Clam Medicine L	6.56	**0.01**
	Clam Rose L	2.7	0.1
	Clam Thompson L	4.8	**0.03**
	Water Bull Run L	7.5	**0.01**
Clam Medicine L	Clam Rose L	7.5	**0.01**
	Clam Thompson L	1.2	0.27
	Water Medicine L	7.5	**0.01**
Clam Rose L	Clam Thompson L	6.82	**0.01**
	Water Rose L	6.82	**0.01**
Clam Thompson L	Water Thompson L	6.82	**0.01**
Water Benewah L	Water Bull Run L	2.45	0.12
	Water Medicine L	0.88	0.35
	Water Rose L	0.01	0.92
	Water Thompson L	0.88	0.35
Water Bull Run L	Water Medicine L	0.53	0.46
	Water Rose L	2.45	0.12
	Water Thompson L	0.1	0.75
Water Medicine L	Water Rose L	0.53	0.46
	Water Thompson L	0.1	0.75
	Water Thompson L	0.53	0.46
Water Rose L	Clam Bull Run L.	2.45	0.12

*Groups that differed significantly from one another are bolded.

Table 10: Pairwise comparisons for Shannon's Diversity (alpha diversity)

Group 1	Group 2	H	p-value*
Clam Benewah L	Clam Bull Run L.	0.23	0.63
	Clam Medicine L	0.23	0.63
	Clam Rose L	0.53	0.47
	Clam Thompson L	0.13	0.72
	Water Benewah L	7.5	**0.01**
Clam Bull Run L.	Clam Medicine L	0.23	0.63
	Clam Rose L	1.2	0.27
	Clam Thompson L	0.53	0.47
	Water Bull Run L	7.5	**0.01**
Clam Medicine L	Clam Rose L	3.33	0.07
	Clam Thompson L	0.53	0.47
	Water Medicine L	7.5	**0.01**
Clam Rose L	Clam Thompson L	0.01	0.92
	Water Rose L	6.82	**0.01**
Clam Thompson L	Water Thompson L	6.82	**0.01**
Water Benewah L	Water Bull Run L	1.84	0.17
	Water Medicine L	0.53	0.46
	Water Rose L	0.01	0.92
	Water Thompson L	0.88	0.35
Water Bull Run L	Water Medicine L	0.1	0.75
	Water Rose L	1.84	0.17
	Water Thompson L	0.1	0.75
Water Medicine L	Water Rose L	0.27	0.6
	Water Thompson L	0.01	0.92
	Water Thompson L	0.88	0.35
Water Rose L	Clam Bull Run L.	1.84	0.17

*Groups that differed significantly from one another are bolded.

Table 11: Pairwise comparisons for Bray-Curtis (beta diversity)

Group 1	Group 2	Pseudo-F	p-value*
Clam Benewah L	Clam Bull Run L.	7.16	**0.002**
	Clam Medicine L	3.64	**0.015**
	Clam Rose L	2.61	**0.062**
	Clam Thompson L	2.48	**0.058**
	Water Benewah L	11.52	**0.001**
Clam Bull Run L.	Clam Medicine L	13.28	**0.004**
	Clam Rose L	6.36	**0.002**
	Clam Thompson L	4.35	**0.003**
	Water Bull Run L	9.1	**0.001**
Clam Medicine L	Clam Rose L	11.65	**0.005**
	Clam Thompson L	3.47	**0.032**
	Water Medicine L	10.28	**0.006**
Clam Rose L	Clam Thompson L	4.37	**0.007**
	Water Rose L	9	**0.006**
Clam Thompson L	Water Thompson L	5.4	**0.008**
Water Benewah L	Water Bull Run L	1.85	0.12
	Water Medicine L	1.49	0.27
	Water Rose L	1.32	0.17
	Water Thompson L	2.07	**0.02**
Water Bull Run L	Water Medicine L	0.77	0.57
	Water Rose L	0.4	0.89
	Water Thompson L	0.22	0.89
Water Medicine L	Water Rose L	0.63	0.77
	Water Thompson L	1.07	0.36
	Water Thompson L	0.48	0.85
Water Rose L	Clam Bull Run L.	1.85	0.12

*Groups that differed significantly from one another are bolded.

Table 12: Pairwise comparisons for Jaccard (beta diversity)

Group 1	Group 2	Pseudo-F	p-value*
Clam Benewah L	Clam Bull Run L.	4	**0.006**
	Clam Medicine L	2.26	**0.016**
	Clam Rose L	2.67	**0.006**
	Clam Thompson L	3.33	**0.007**
	Water Benewah L	7.63	**0.006**
Clam Bull Run L.	Clam Medicine L	5.95	**0.003**
	Clam Rose L	2.29	**0.024**
	Clam Thompson L	4.97	**0.003**
	Water Bull Run L	5.36	**0.003**
Clam Medicine L	Clam Rose L	5.77	**0.003**
	Clam Thompson L	3.61	**0.007**
	Water Medicine L	8.38	**0.002**
Clam Rose L	Clam Thompson L	4.76	**0.008**
	Water Rose L	5.09	**0.006**
Clam Thompson L	Water Thompson L	4.98	**0.011**
Water Benewah L	Water Bull Run L	1.37	0.152
	Water Medicine L	1.15	0.266
	Water Rose L	1.1	0.275
	Water Thompson L	1.55	**0.019**
Water Bull Run L	Water Medicine L	0.94	0.481
	Water Rose L	0.72	0.773
	Water Thompson L	0.55	1
Water Medicine L	Water Rose L	0.89	0.524
	Water Thompson L	1.12	0.34
	Water Thompson L	0.73	0.835
Water Rose L	Clam Bull Run L.	4	0.006

*Groups that differed significantly from one another are bolded.

Table 13: Pairwise comparisons for weighted UniFrac (beta diversity)

Group 1	Group 2	Pseudo-F	p-value*
Clam Benewah L	Clam Bull Run L.	5.88	**0.002**
	Clam Medicine L	1.19	0.336
	Clam Rose L	4.31	**0.017**
	Clam Thompson L	1.25	0.324
	Water Benewah L	20.2	**0.002**
Clam Bull Run L.	Clam Medicine L	8.2	**0.002**
	Clam Rose L	7.55	**0.014**
	Clam Thompson L	3.86	**0.015**
	Water Bull Run L	15.89	**0.003**
Clam Medicine L	Clam Rose L	5.41	**0.008**
	Clam Thompson L	2.18	0.109
	Water Medicine L	22.24	**0.002**
Clam Rose L	Clam Thompson L	3.64	**0.041**
	Water Rose L	32.95	**0.015**
Clam Thompson L	Water Thompson L	7.78	**0.01**
Water Benewah L	Water Bull Run L	1.25	0.38
	Water Medicine L	1.66	0.28
	Water Rose L	0.94	0.42
	Water Thompson L	1.66	0.24
Water Bull Run L	Water Medicine L	1.09	0.47
	Water Rose L	0.46	0.71
	Water Thompson L	0.11	0.97
Water Medicine L	Water Rose L	0.56	0.61
	Water Thompson L	1.5	0.20
	Water Thompson L	0.51	0.66
Water Rose L	Clam Bull Run L.	1.25	0.38

*Groups that differed significantly from one another are bolded.

Table 14: Pairwise comparisons for unweighted UniFrac (beta diversity)

Group 1	Group 2	Pseudo-F	p-value*
Clam Benewah L	Clam Bull Run L.	3.86	**0.006**
	Clam Medicine L	3.39	**0.009**
	Clam Rose L	2.33	**0.031**
	Clam Thompson L	4.24	**0.007**
	Water Benewah L	17.85	**0.002**
Clam Bull Run L.	Clam Medicine L	9.37	**0.002**
	Clam Rose L	3.05	**0.008**
	Clam Thompson L	6.34	**0.004**
	Water Bull Run L	9.02	**0.005**
Clam Medicine L	Clam Rose L	7.55	**0.002**
	Clam Thompson L	4.14	**0.005**
	Water Medicine L	14.06	**0.001**
Clam Rose L	Clam Thompson L	11.9	**0.002**
	Water Rose L	5.9	**0.013**
Clam Thompson L	Water Thompson L	8.52	**0.006**
Water Benewah L	Water Bull Run L	1.55	0.15
	Water Medicine L	1.29	0.19
	Water Rose L	1.08	0.3
	Water Thompson L	1.5	0.09
Water Bull Run L	Water Medicine L	0.76	0.62
	Water Rose L	0.72	0.73
	Water Thompson L	0.5	0.98
Water Medicine L	Water Rose L	0.72	0.71
	Water Thompson L	0.99	0.4
	Water Thompson L	0.67	0.82
Water Rose L	Clam Bull Run L.	1.55	0.15

*Groups that differed significantly from one another are bolded.

Table 15: Clam sample alpha diversity means

Metric	Rose L.	BR L.	Medicine L.	Thompson L.	Benewah L.
Obs. OTUs	20.4 [3.78]	19.83 [3.65]	14.17 [2.99]	13.6 [4.56]	18.67 [4.72]
Pielou's Evn.	0.5 [0.03]	0.57 [0.11]	0.69 [0.06]	0.59 [0.15]	0.55 [0.07]
Faith's PD	4.94 [0.77]	4.22 [0.50]	3.23 [0.36]	3.03 [0.64]	3.96 [0.48]
Shannon	2.16 [0.21]	2.44 [0.57]	2.45 [0.23]	2.21 [0.65]	2.32 [0.35]

Data presented as mean [SD].

Table 16. Water sample alpha diversity means

Metric	Rose L.	BR L.	Medicine L.	Thompson L.	Benewah L.
Obs. OTUs	155.4 [67.77]	117 [41.97]	132 [46.99]	130.2 [80.82]	149.4 [38.23]
Pielou's Evn.	0.81 [0.05]	0.76 [0.04]	0.76 [0.06]	0.77 [0.09]	0.79 [0.04]
Faith's PD	12.97 [4.06]	9.38 [3.77]	11.20 [3.90]	10.94 [5.69]	13.15 [3.30]
Shannon	5.83 [0.91]	5.20 [0.60]	5.34 [0.85]	5.31 [1.29]	5.69 [0.58]

Data presented as mean [SD].

FIGURES AND FIGURE LEGENDS

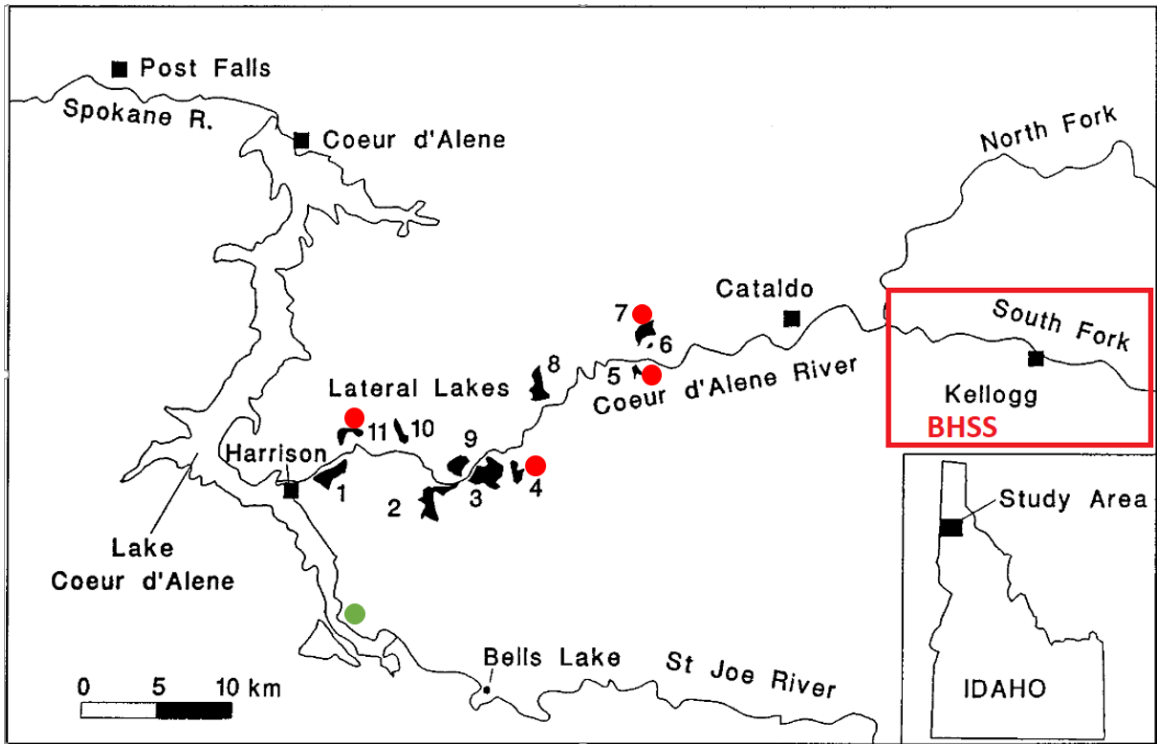

Figure 1. Map of sampling locations. Sampled water bodies are marked in red: 7, Rose L.; 5, Bull Run L.; 4, Medicine L.; 11, Thompson L. Benewah L., the reference site, is marked in green. The Bunker Hill Superfund Site is outlined with a red box. Arrows indicate the direction of water flow. Map adapted from (Sprenke et al, 2000).

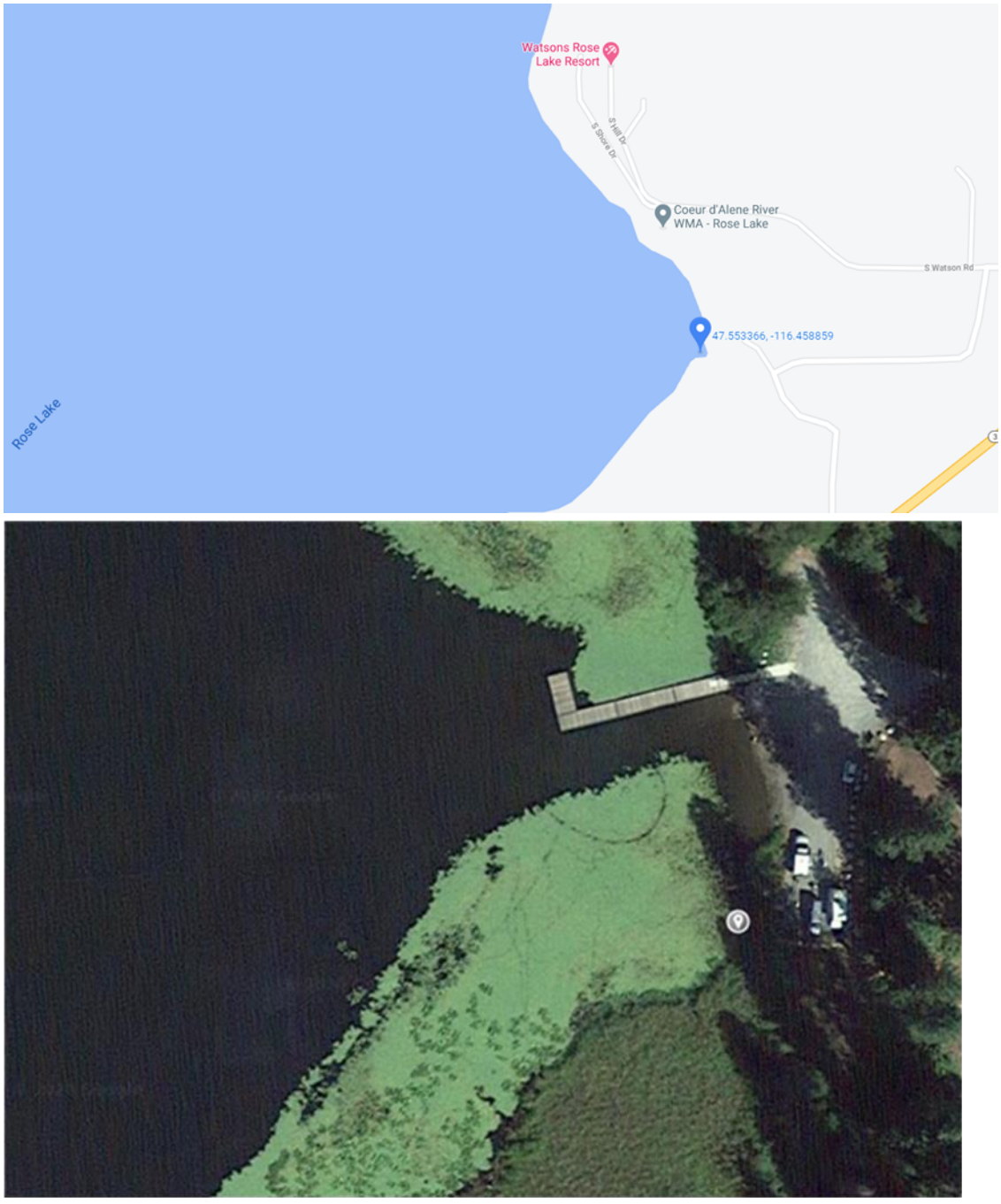

Figure 2. Rose L. collection site. The collection site was adjacent to the public fishing dock, accessed from the unmarked rod that branches off of S. Watson Rd, comparable to site S2 in Higbee (2017). The collection site is noted with the blue marker and coordinates in the top picture and the gray marker in the bottom picture. All pictures from Google Maps.

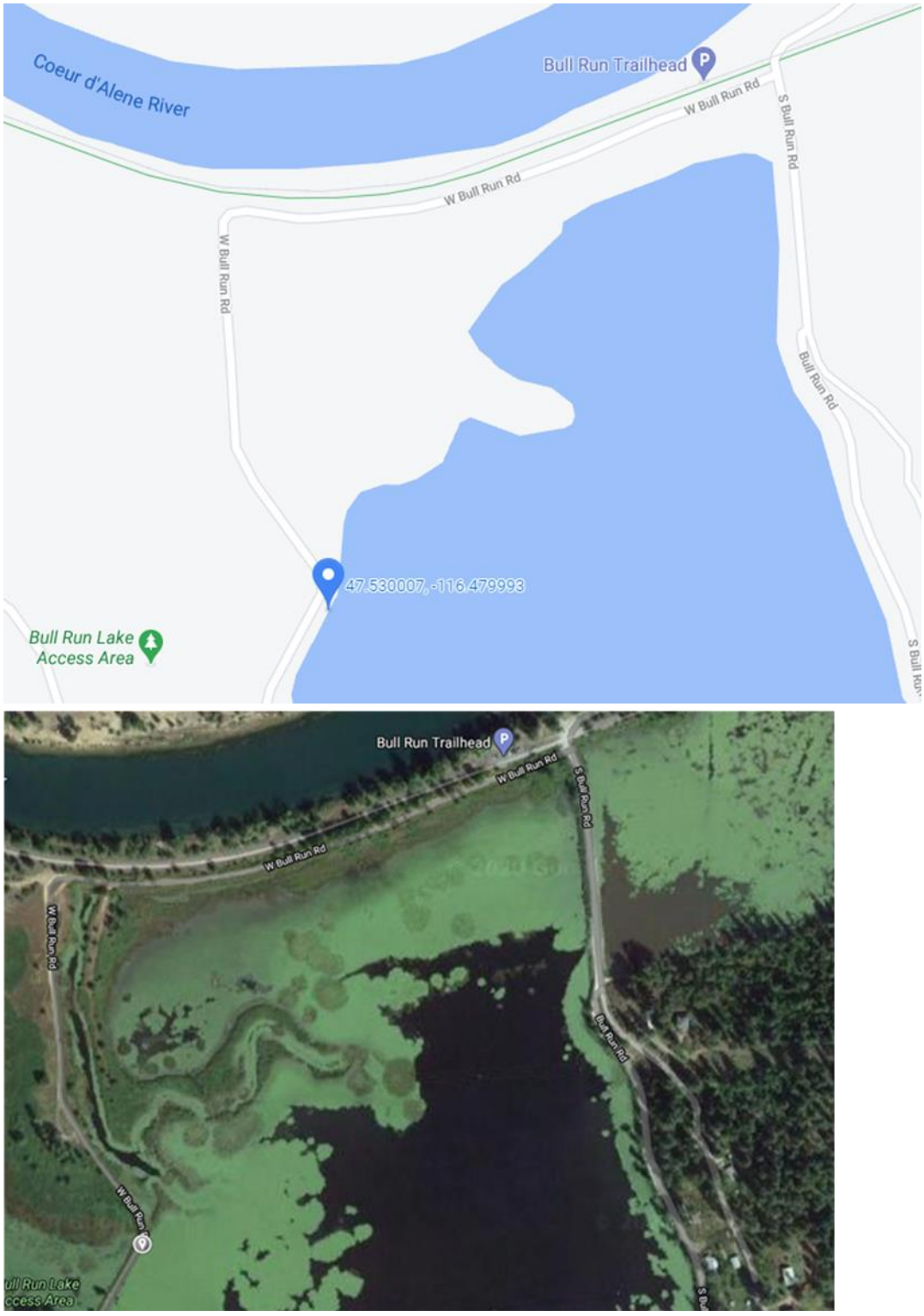

Figure 3. Bull Run L. collection site. The collection site was along the edge of the W Bull Run Rd, slightly north of site S1 in Higbee (2017). The collection site is noted with the blue marker and coordinates in the top picture and the gray marker in the bottom picture.

Figure 4. Medicine L. collection site. The collection site was along the edge of the peninsula adjacent to the public parking off of E. Rain Hill Rd., comparable to site S1 in Higbee (2017). The collection site is noted with the blue marker and coordinates in the top picture and the gray marker in the bottom picture.

Figure 5. Thompson L. collection site. The collection site was accessed from the public parking near where E. Thompson Lake Rd and S. Ashbury Rd intersect, comparable to site S3 in Higbee (2017). As this site is adjacent to the main lakebed, two pictures are provided to illustrate the site. The collection site is noted with the blue marker and coordinates in the top picture and the gray marker in the middle and bottom pictures.

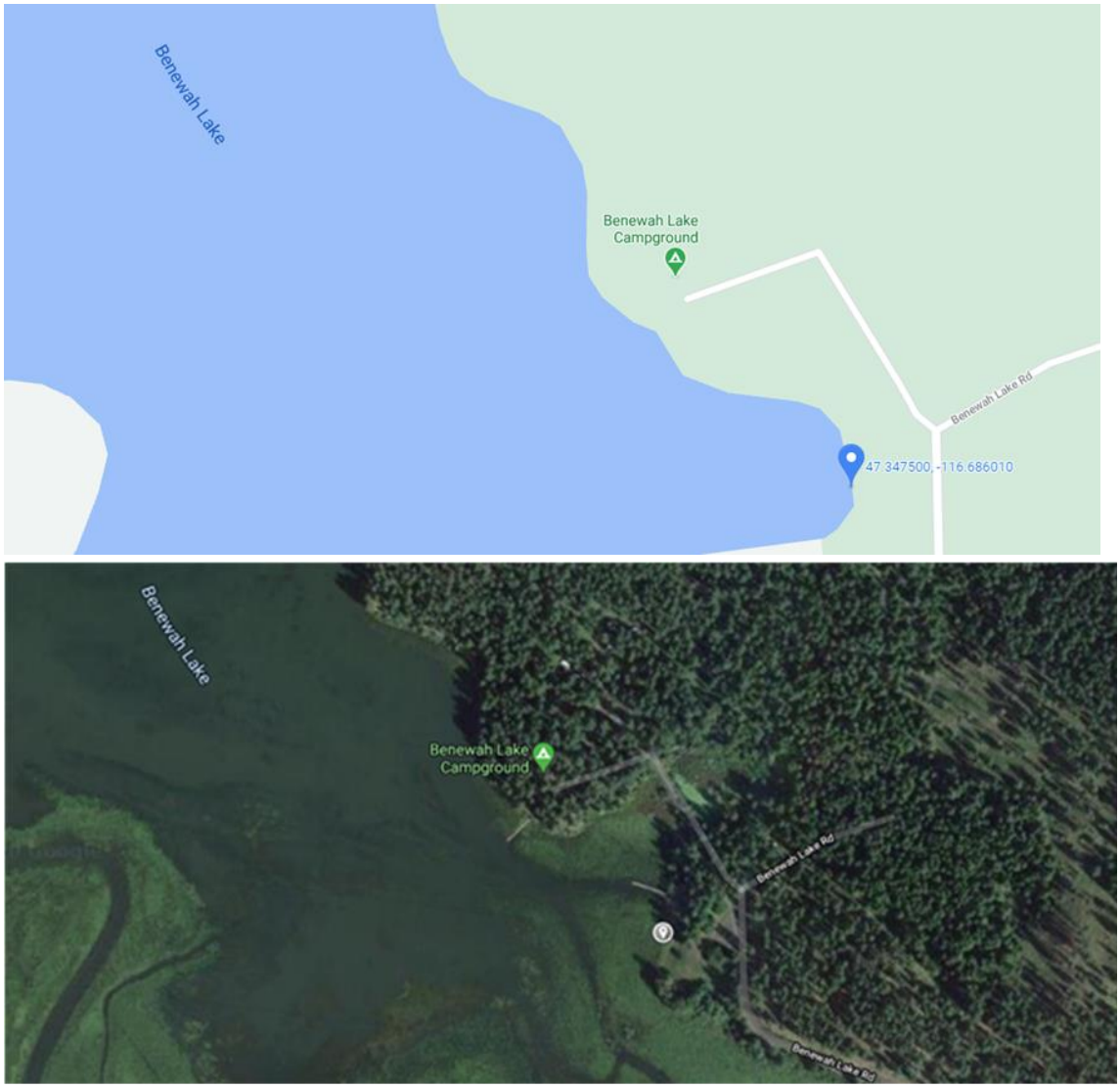

Figure 6. Benewah L. collection site. The collection site was accessed from the public parking lot by the fishing dock for the Benewah L Campground, off of Benewah Lake Rd, comparable to site S2 in Higbee (2017). The collection site is noted with the blue marker and coordinates in the top picture and the gray marker in the bottom picture. This site is accessed from Heyburn State Park but as it lies within the boundaries of the Coeur D'Alene Tribe reservation, we sampled with permission from the Tribe.

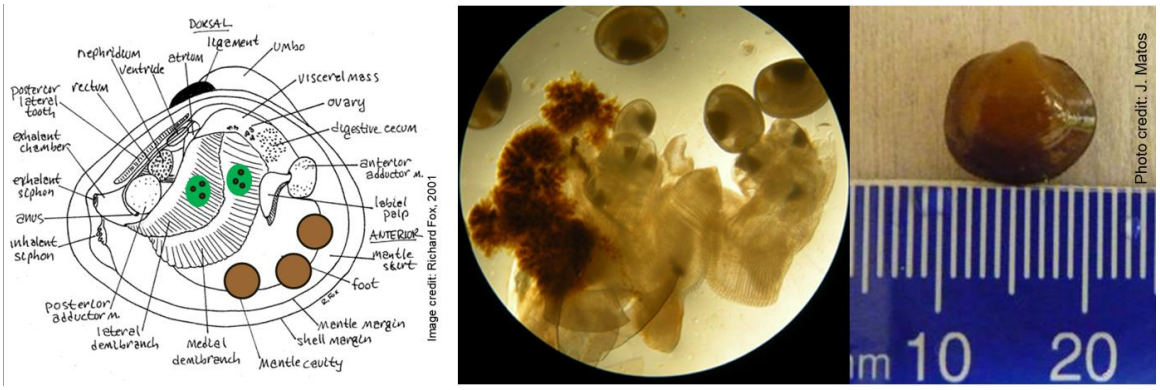

Figure 7. Clam anatomy and brood. L-R: clam anatomy with brood pouches (green) in gills, shelled juveniles (brown) in mantle cavity; shelled juveniles (large), larvae (small), partial brood pouch; mature clam with approximately a 9 mm shell length.

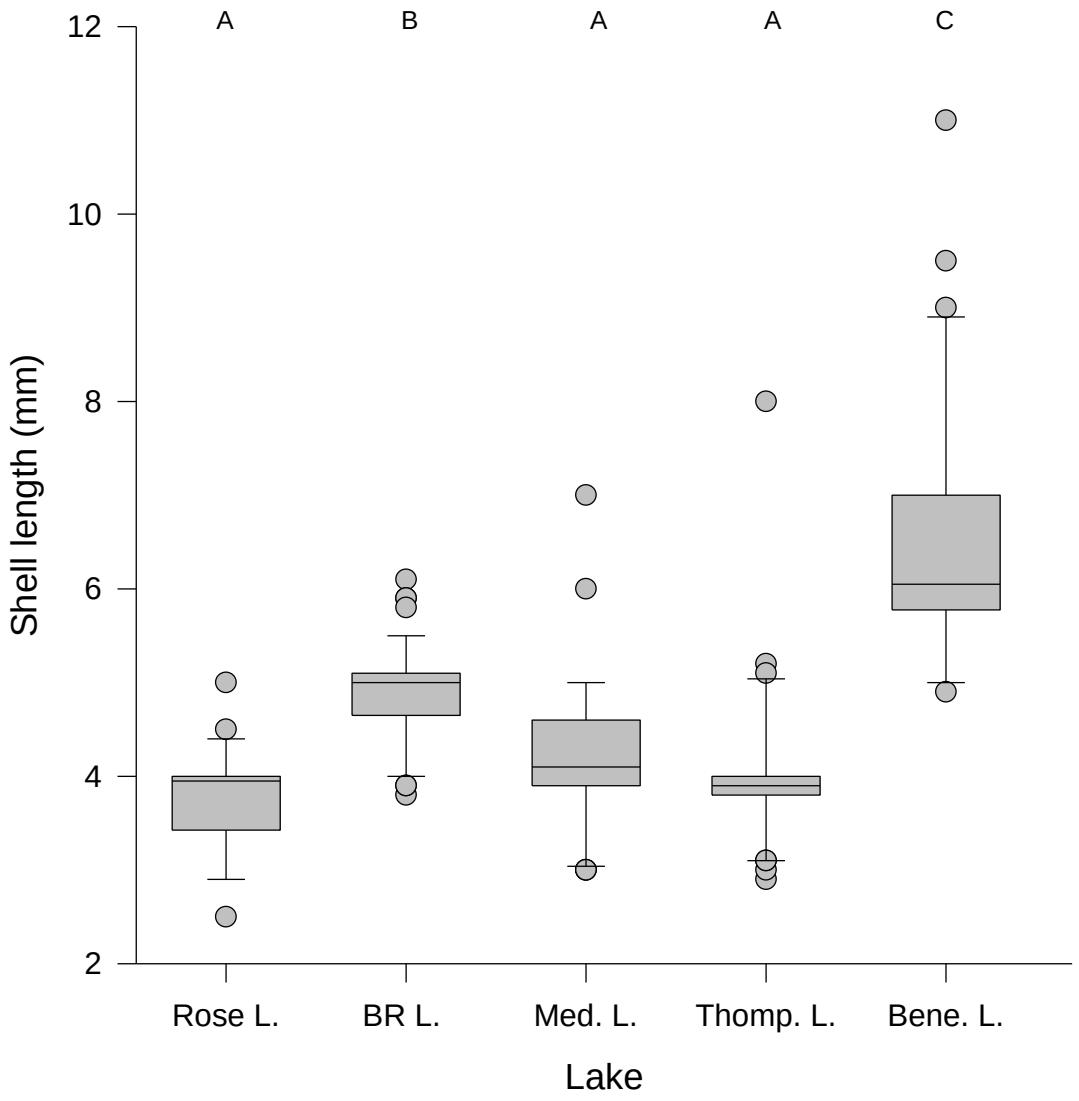

Figure 8. Shell length comparisons across the five clam populations. Data sets with different letters indicate statistically significant differences (Kruskal-Wallis ANOVA, $p < 0.001$; multiple comparisons, $p < 0.05$). Samples sizes in Table 5. BR, Bull Run L., Med., Medicine L., Thomp., Thompson L., Bene., Benewah L.

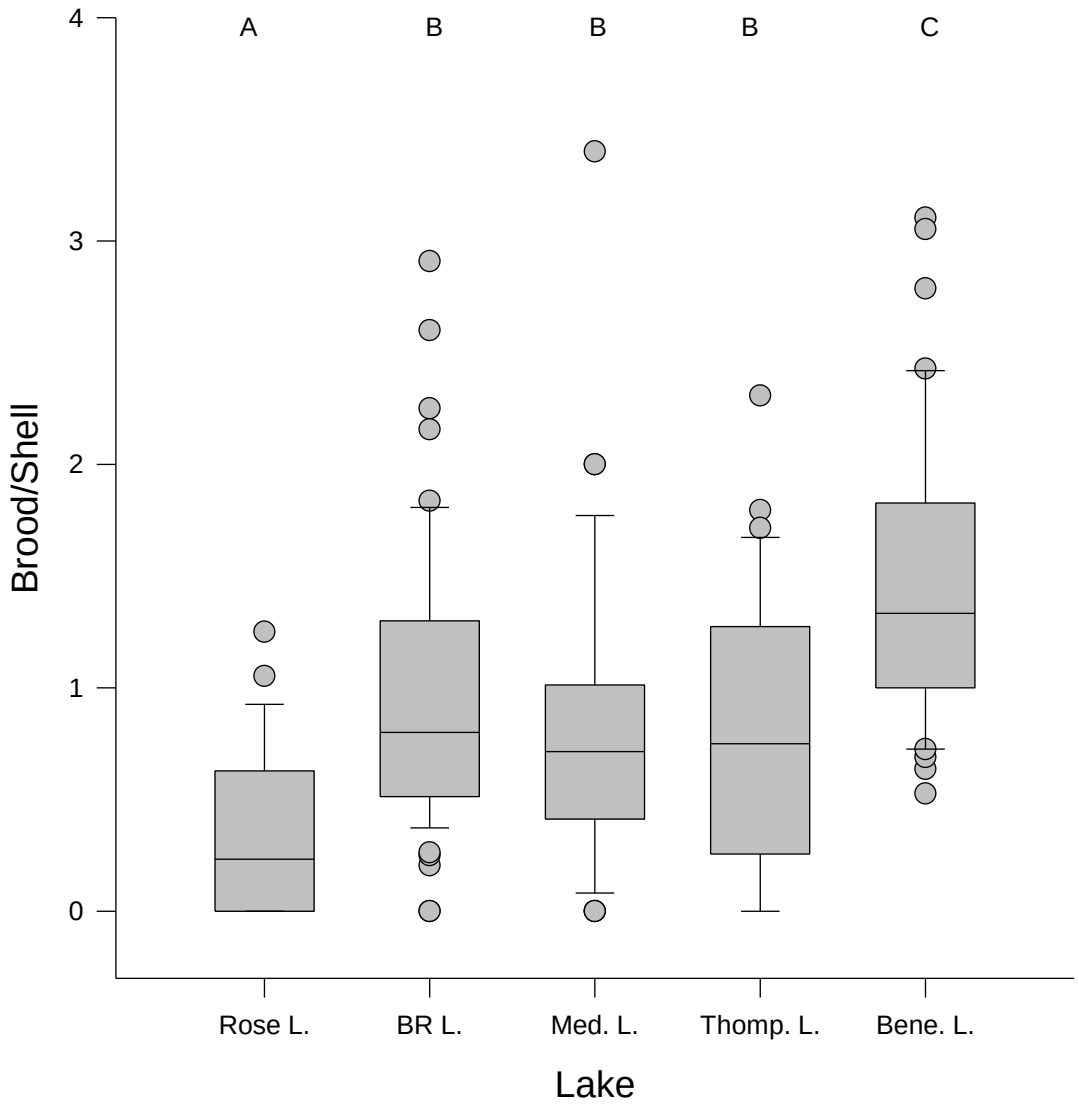

Figure 9. Total reproductive output of the five clam populations. Reproductive output is calculated as brood/shell length Data sets with different letters indicate statistically significant differences (Kruskal-Wallis ANOVA, $p < 0.001$; multiple comparisons, $p < 0.05$). BR, Bull Run L., Med., Medicine L., Thomp., Thompson L., Bene., Benewah L.

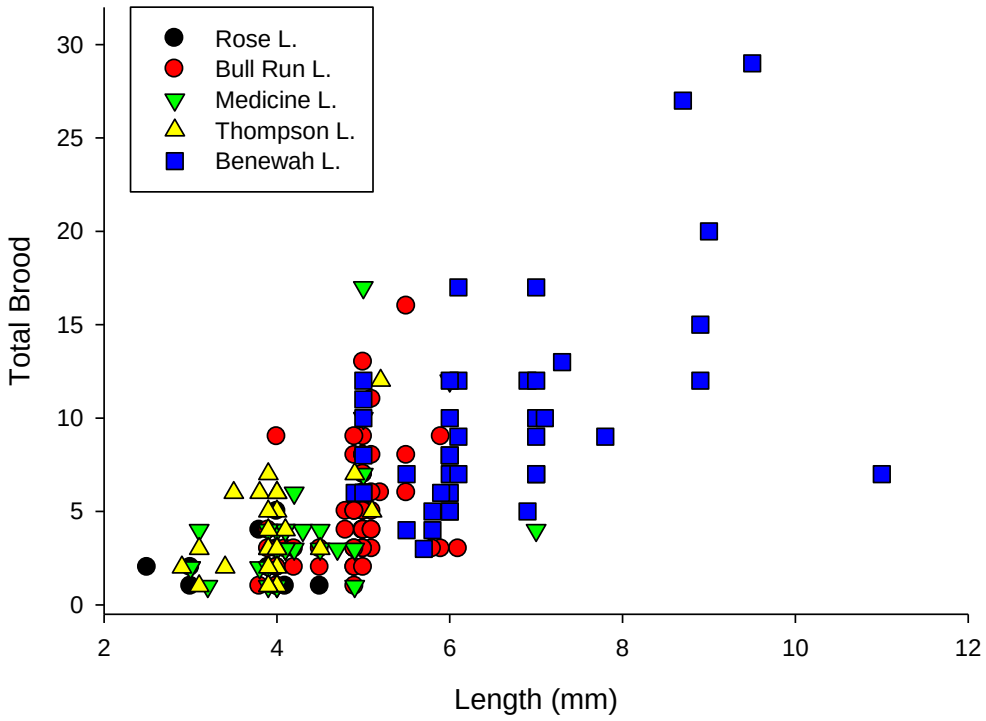

Figure 10. Relationship between total brood and shell length for all brooding clams. Only those clams that were brooding are included. Linear regression, $R^2 = 0.48$, $p < 0.001$; analysis performed on square-root transformed data, untransformed data shown here)

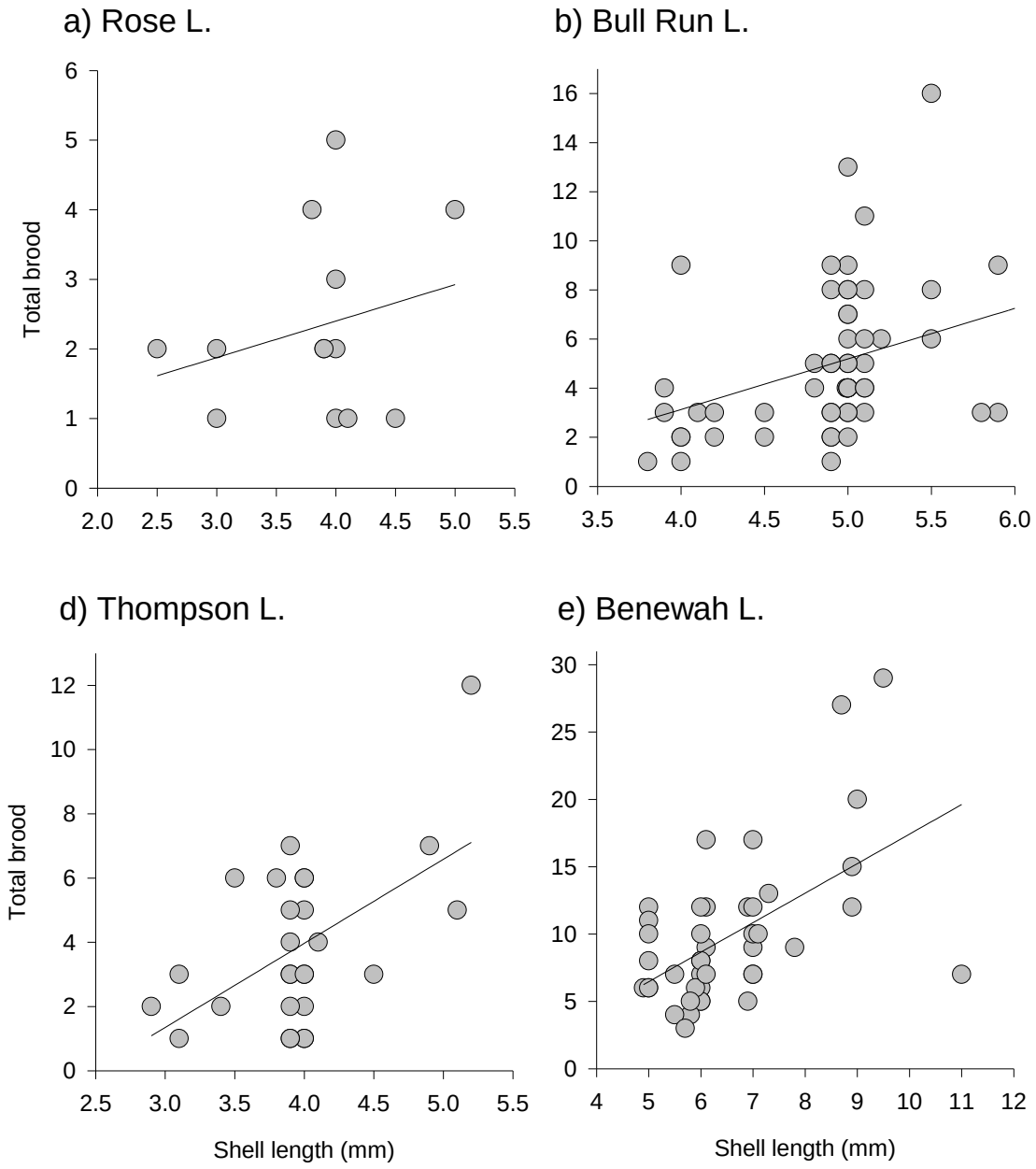

Figure 11. Relationship between total brood and shell length in brooding clams. Medicine lake clams are not represented as the regression failed assumptions. Square root transformed data were analyzed by linear regression; untransformed data are represented here. Rose L., R^2 = 0.05, p = 0.47; Bull Run L., R^2 = 0.15, p = 0.003; Thompson L. , R^2 = 0.25, p = 0.009; Benewah L., R^2 = 0.29, p < 0.001.

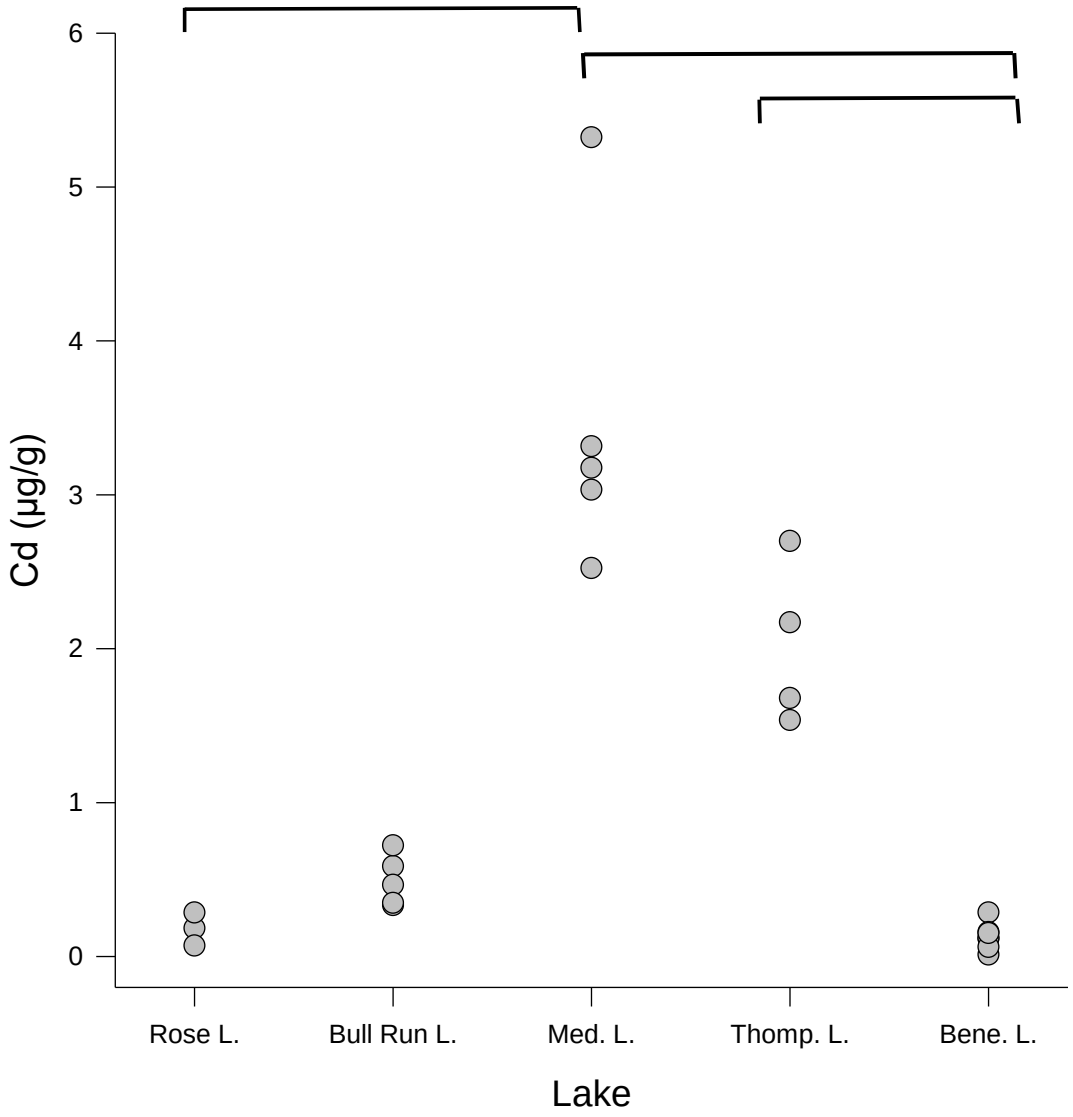

Figure 12. Tissue Cd levels of pooled clam samples from each population. Each pooled sample contained three clams; sample size ranged from 3-7 samples per lake (Table 6). Samples connected by brackets are significantly different (Kruskal-Wallis ANOVA, $p < 0.001$; multiple comparisons, $p < 0.05$). BR, Bull Run L., Med., Medicine L., Thomp., Thompson L., Bene., Benewah L.

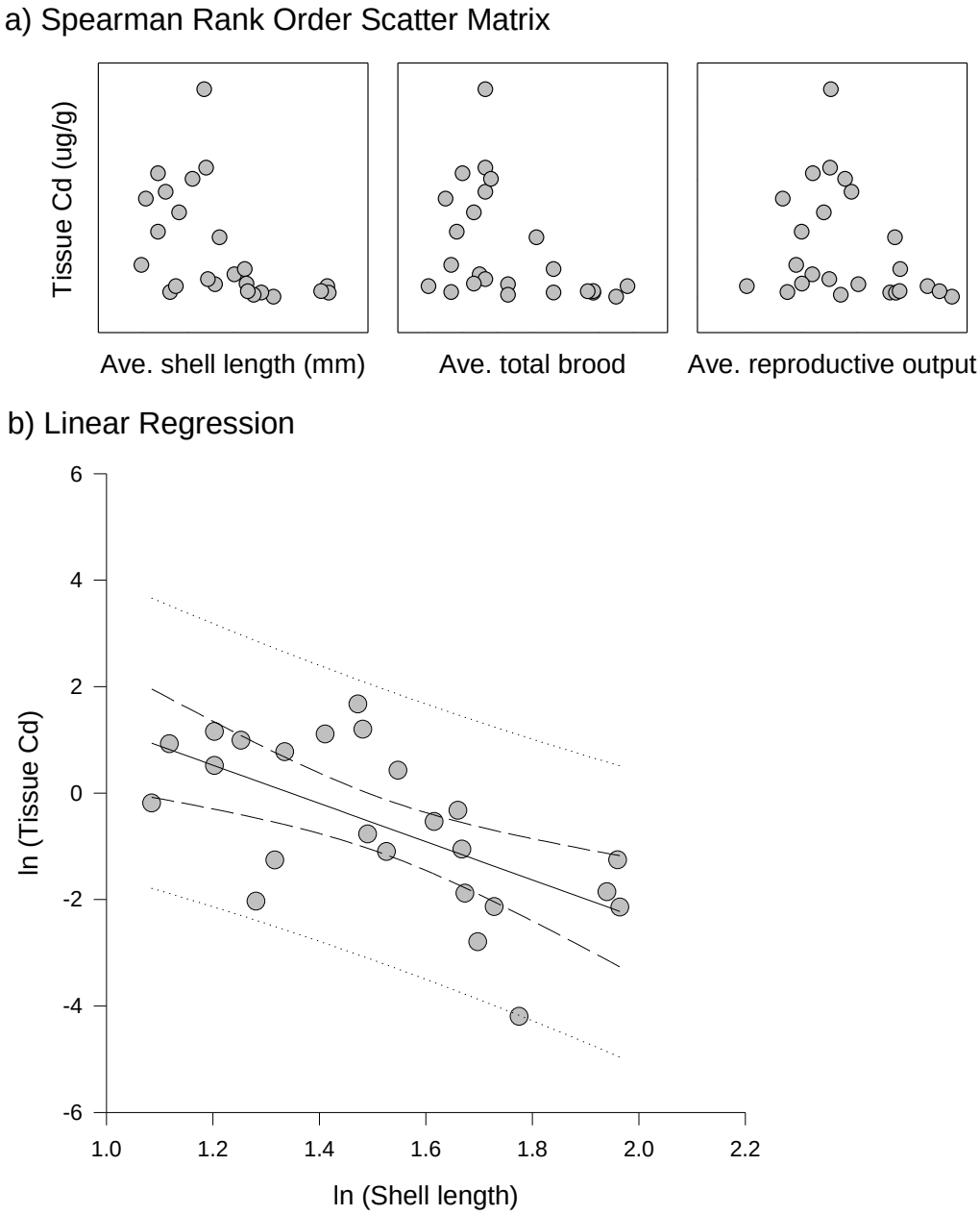

Figure 13. Spearman Rank Order correlation between clam tissue metal content, total brood, shell length, and reproductive output. Tissue Cd has a negative relationship to shell length (R^2 = 0.38, p = 0.001) and is inversely related to average total brood count (rs -0.449, p = 0.027).

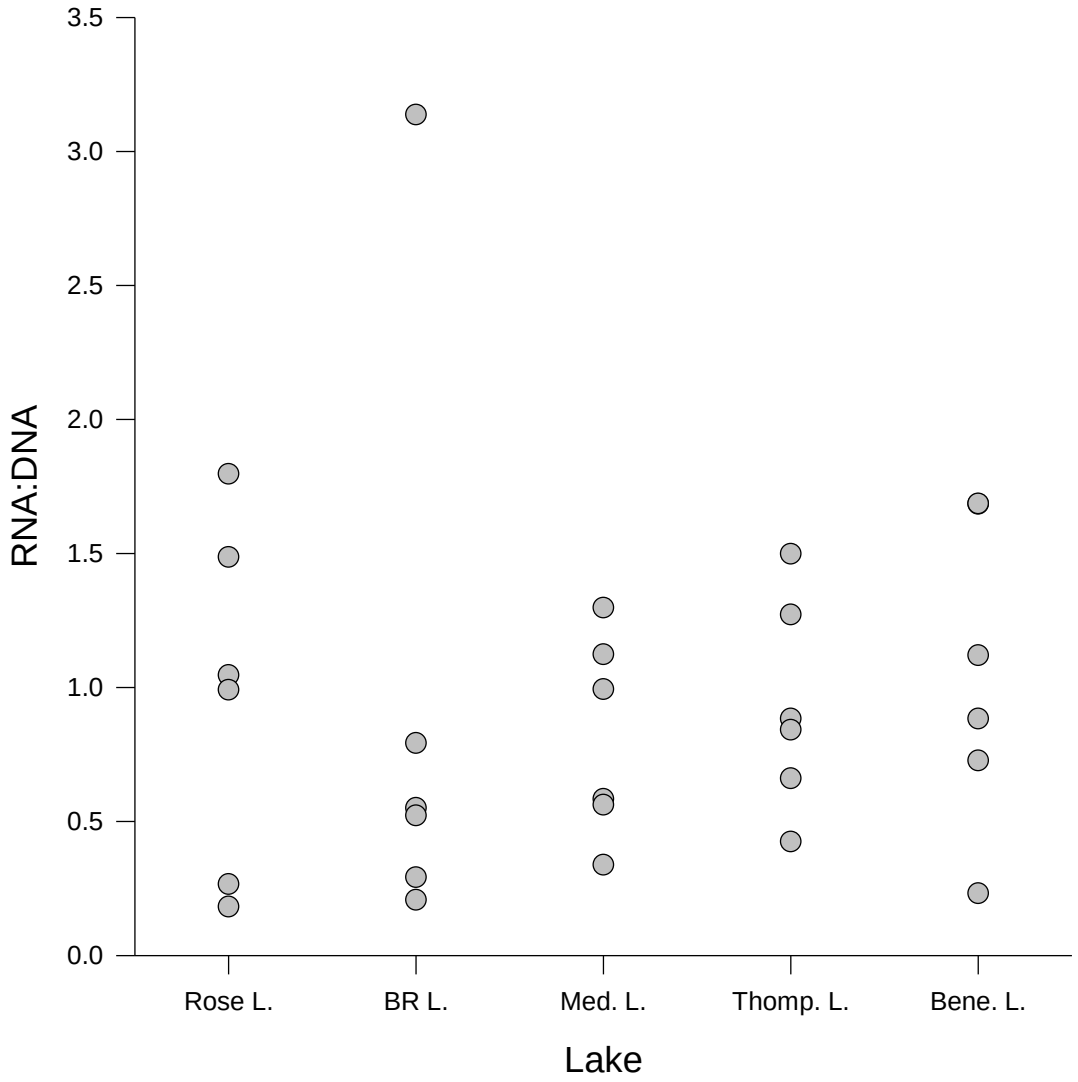

Figure 14. RNA:DNA of individual clams from each population. Sample size is six clams per lake. RNA:DNA was determined for clams used in the microbiome analysis. There were no differences across groups ($p = 0.734$). BR, Bull Run L., Med., Medicine L., Thomp., Thompson L., Bene., Benewah L.

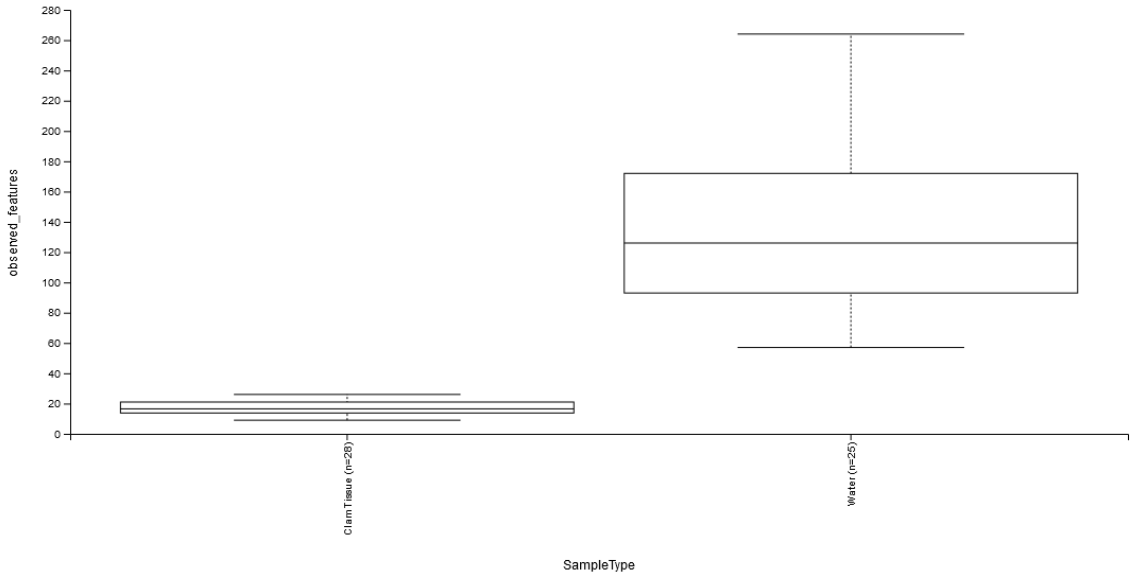

Figure 15. Boxplot showing alpha diversity among the clam and lake samples. L-R: Clam samples, water samples. This boxplot displays the difference in Observed Features/OTUs between groups. The two groups were significantly different (Kruskal-Wallis ANOVA, $p < 0.001$).

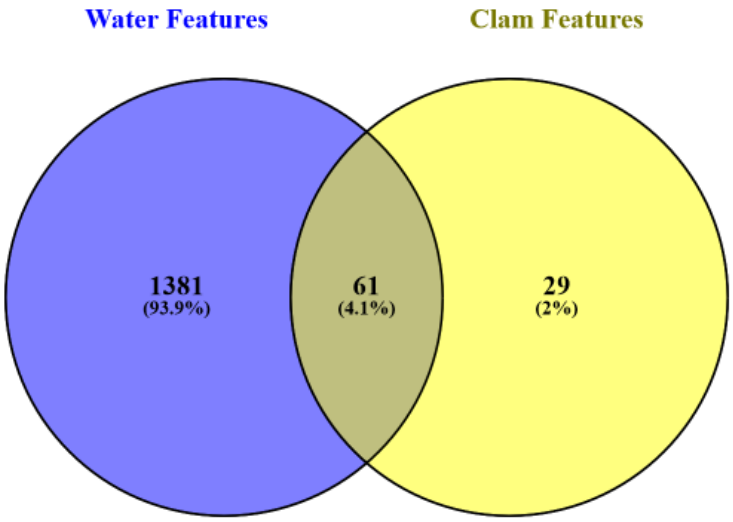

Figure 16. Venn diagram showing percentages of water and clam features. Blue indicates features present only in water samples (1,381), yellow indicates features that were present only in clam samples (29), and the shaded area indicates common features between the two (61).

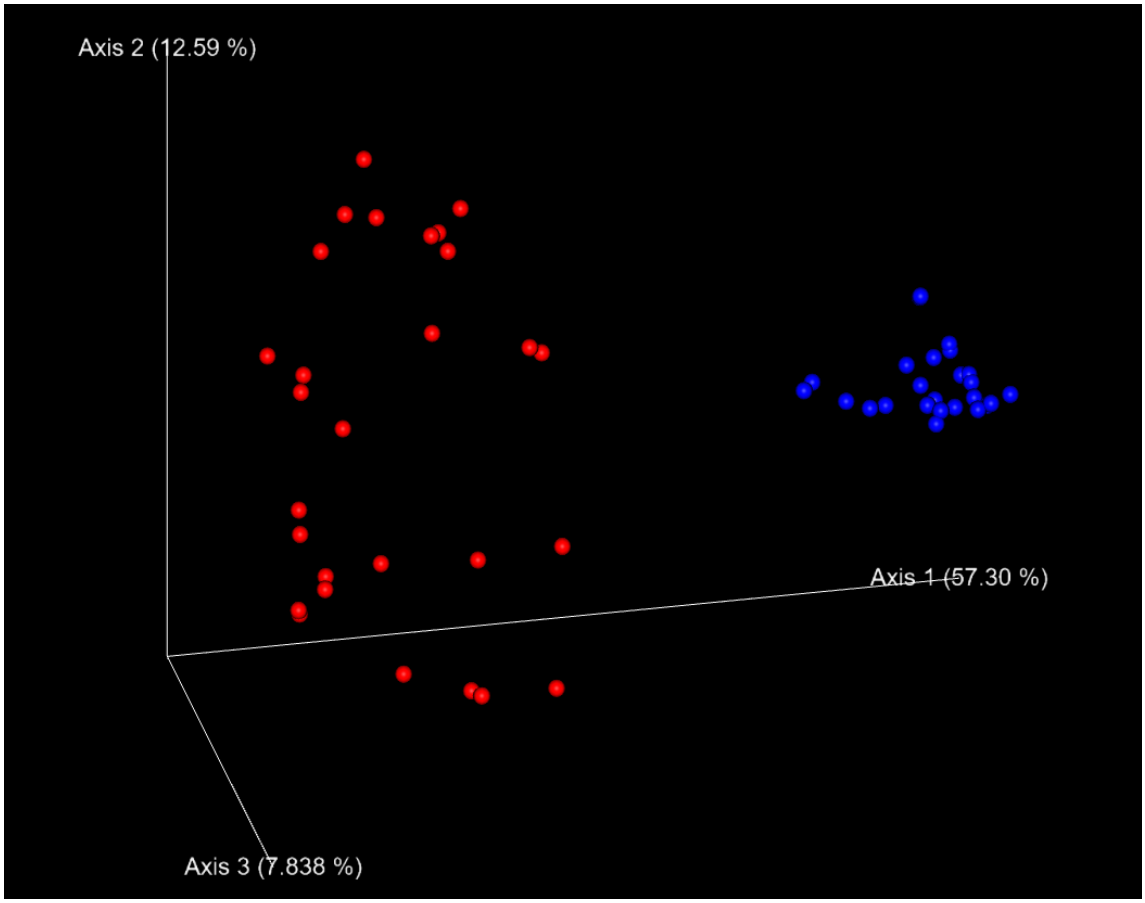

Figure 17. Principal Coordinates Analysis (PCoA) ordination on Weighted UniFrac distance matrix of all samples. Clam tissue samples are indicated in red, water samples are indicated in blue.

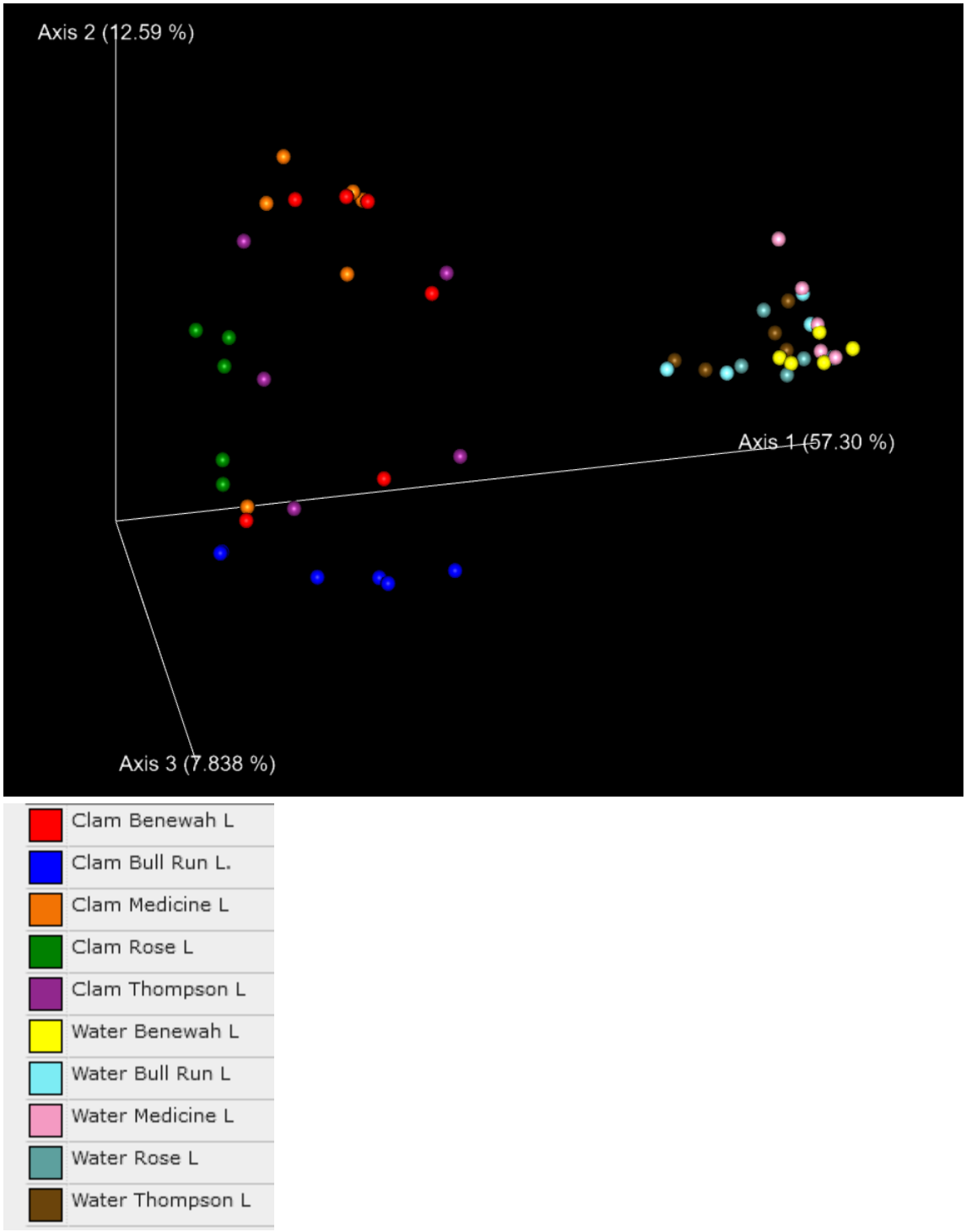

Figure 18. Principal Coordinates Analysis (PCoA) ordination on Weighted UniFrac distance matrix of all samples, colored by the variable SampleTypeSite.

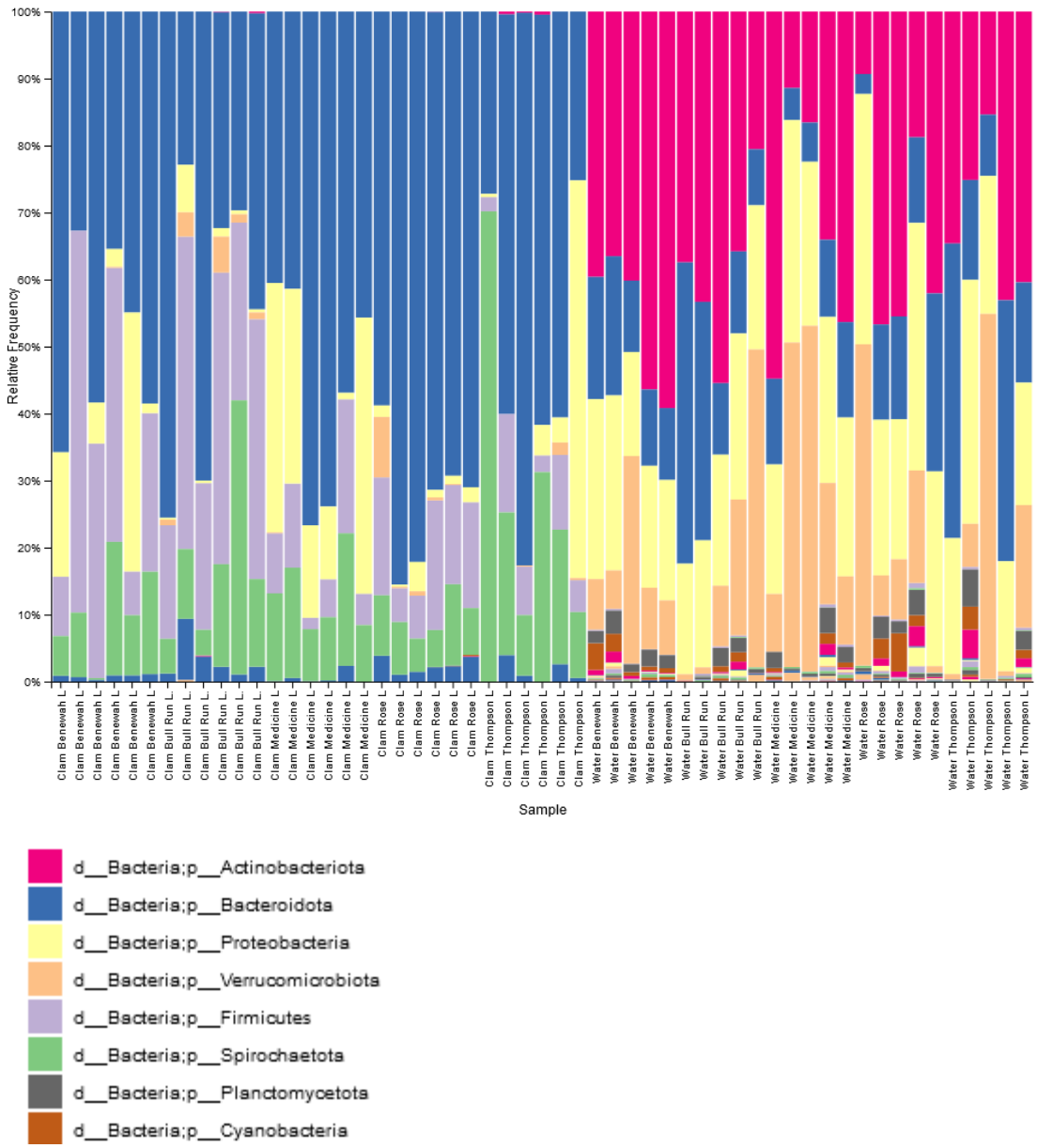

Figure 19. Taxonomical barplot of bacterial phyla found in clam and water samples. Legend shown is representative of bars above 10% of relative frequency. Coloration not representative of the same taxa as other barplot figures.

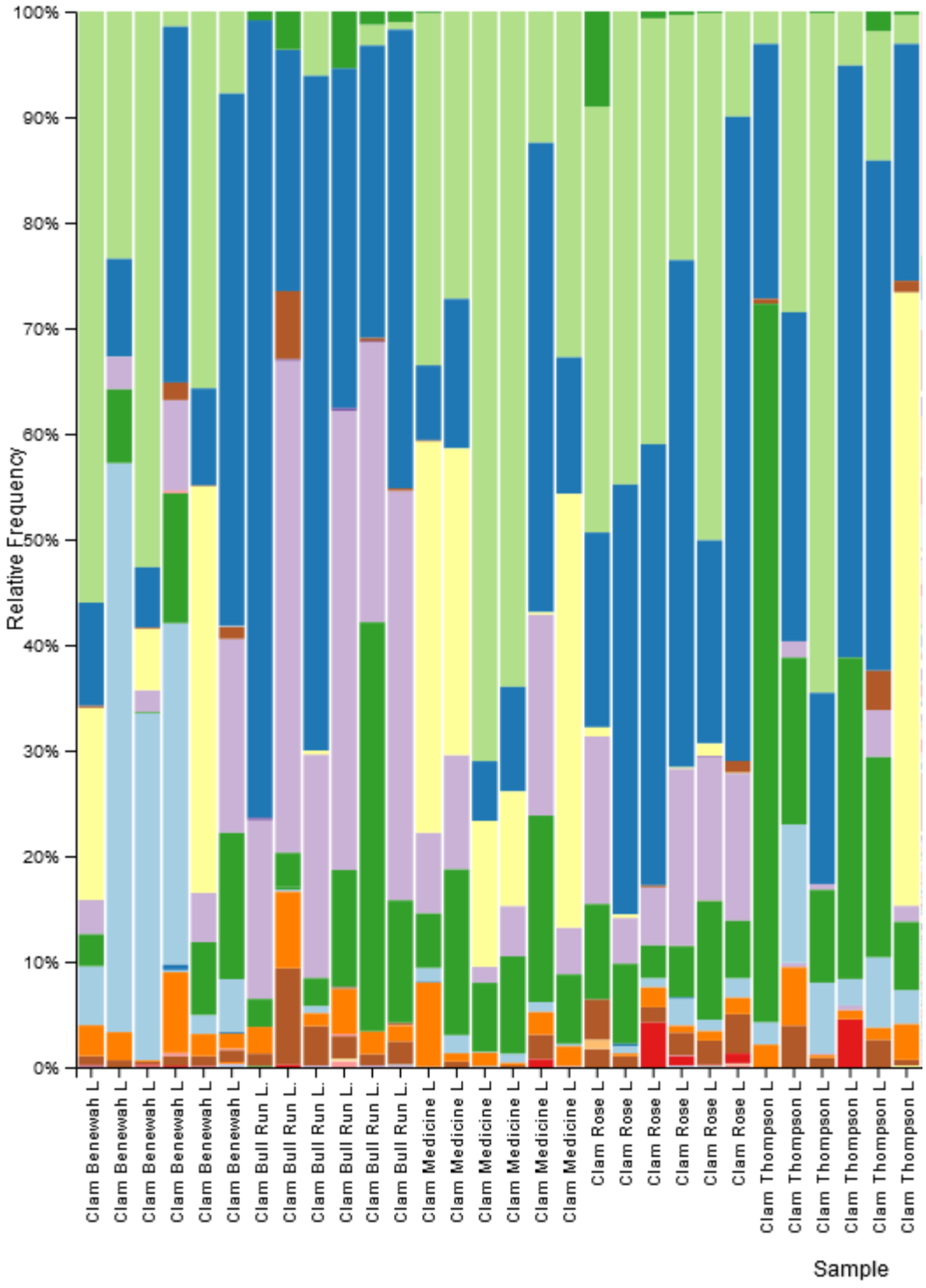

Figure 20. Taxonomical barplot of clam samples. Bacteria identified to the genus level. Legend (Figure 22) is different from legend for Figure 20.

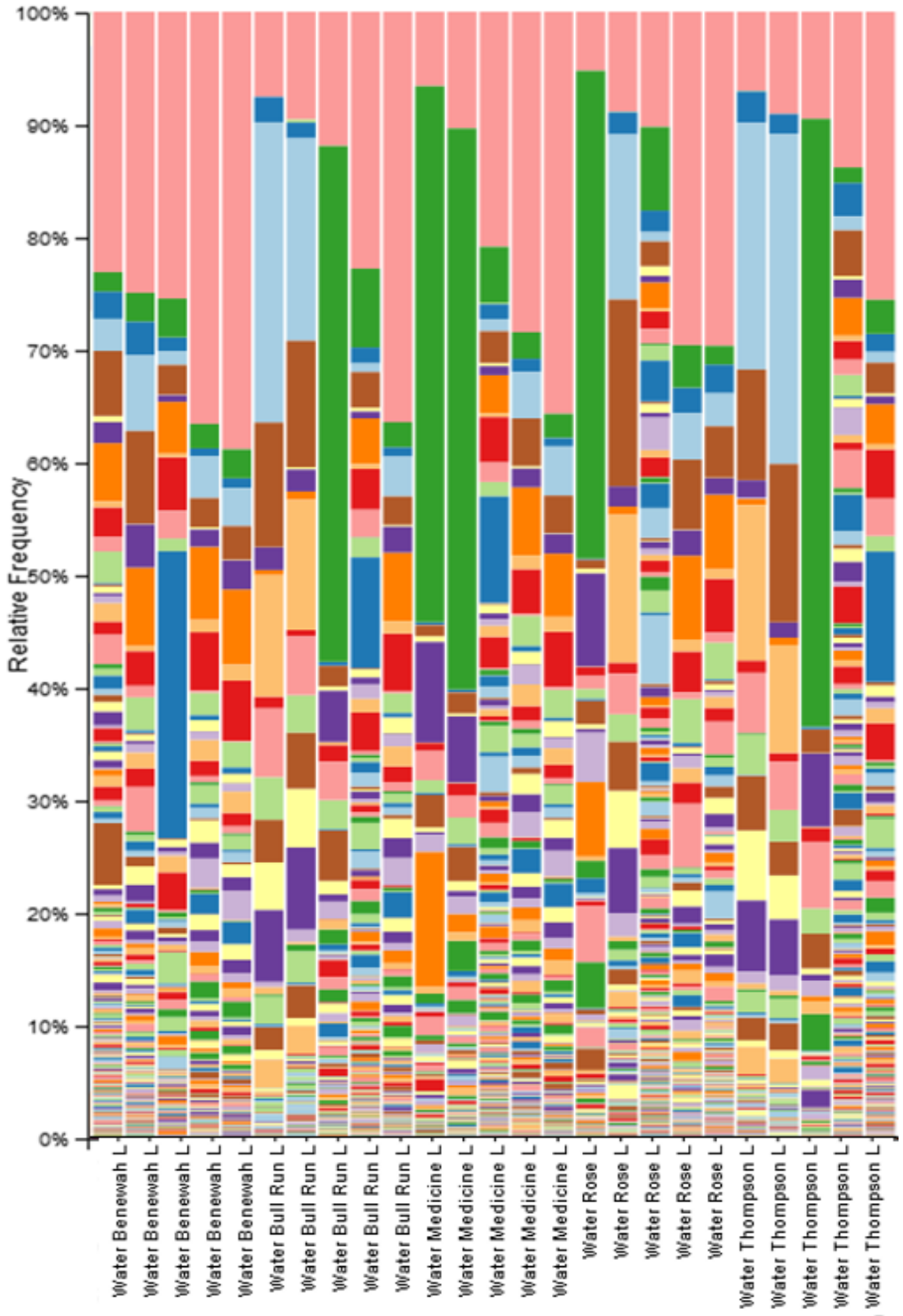

Figure 21. Taxonomical barplot of water samples. Bacteria identified to genus level. Legend (Figure 22) is different from legend for Figure 20.

Figure 22. Legend for Figures 20, 21.

APPENDIX

Protocol 1: YSI storage/setup and recalibration protocol.
Note: this is *not* the “first time setup” protocol in the manual, these are from the calibration sections.
Information taken directly from user manual. Probe identification and placement depends unit to unit and use to use; refer to manual and look at the unit to identify all relevant parts before proceeding.

Long term storage- temperature

No special storage required. Remove probe from unit and store in labeled ziplock bag.

Long term storage- conductivity

No special storage required. Remove probe from unit and store in labeled ziplock bag.

Long term storage- DO

1. Remove membrane cap
2. Rinse sensor with clean water (we used DI)
3. Allow probe to air dry
4. Install clean, new, dry membrane over sensor. The membranes came with the unit, in cardboard boxes, and are the yellow plastic caps that go over the ends of the sensors.
5. Put away in bag.

Long term storage- pH

1. Remove from cable, fill vacant port with plug if you have it. Some kits may or may not include them (ours did not)
2. Make sure the original shipping bottle is full with buffer 4 solution from YSI kit
3. Submerge sensor in solution. Store in a way where the sensor remains immersed.

To ‘wake up’ the YSI for a new field season, the probes need specific steps for recalibration.

Setup- conductivity

1. Turn unit on (power button.) Press Cal. Highlight Probe ID or User ID. User ID will appear automatically. Select ‘None’ if you do not want a User ID stored with the calibration.
2. After selecting the User ID and/or Probe ID if appropriate, highlight Conductivity and press enter.
3. Highlight the desired calibration method; Sp. Conductance, Conductivity, or Salinity and press enter.
(We used Sp. Conductance using the provided calibration solution in the kit.)4. Pour some fresh, traceable conductivity calibration solution into a beaker. No specific volume- pour enough so that when the probe and unit are dipped in the solution covers the holes of the conductivity sensor that are closest to the cable.
Ensure the entire conductivity sensor is submerged in the solution or the instrument will read approximately of half the expected value.
5. Choose the units in either SPC-us/cm, C-us/cm or SPC-ms/cm, C-ms/cm and press enter.
6. Highlight Calibration value and press enter to input the value of the calibration standard (information should come from the label of the bottle that came in the kit.

Then, once the temperature and conductivity readings stabilize, highlight Accept Calibration and press enter.
After completing the calibration, the message line at the bottom of the screen will display "Calibrating Channel..." and then "Saving Configuration...".

Setup-pH

1. Get out bottles of the 3 pH solutions that came with the YSI kit. These are your 3 calibration points.

2. Press Cal. Highlight Probe ID or User ID. User ID will appear automatically. Select 'None' if you do not want a User ID stored with the calibration.
3. After selecting the User ID and/or Probe ID if appropriate, highlight ISE (pH) and press enter.
4. The message line will show the instrument is "Ready for point 1".
5. Place the sensor in your first YSI pH buffer solution. The instrument should automatically recognize the buffer value and display it at the top of the calibration screen. If the calibration value is incorrect, the auto buffer recognition setting in the Sensor Setup menu may be incorrect. If necessary, highlight the Calibration Value and press enter to input the correct buffer value.
6. Once the pH and temperature readings stabilize, highlight Accept Calibration and press enter to accept the first calibration point. The message line will then display "Ready for point 2".
7. To continue with the 2nd point, place the sensor in the second buffer solution. The instrument should automatically recognize the second buffer value and display it at the top of the screen. If necessary, highlight the Calibration Value and press enter to input the correct buffer value.
8. Once the pH and temperature readings stabilize, highlight Accept Calibration and press enter to confirm the second calibration point. The message line will then display 'Ready for point 3".
9. You can continue with the 3rd calibration point in this same method if desired.
10. Continue in this fashion until the desired number of calibration points is achieved (we did 3 for the 3
bottles of solution.)
11. When done, press Cal to complete the calibration.

Setup- DO

I did Calibrating DO % in Water Saturated Air: 1-Point Calibration

1. The supplied sensor storage container (screw on plastic cup for the dual-port and Quatro cables) can be used for DO calibration purposes.)
2. Moisten the sponge in the storage sleeve or plastic cup with a small amount of clean tap water. The sponge should be clean since bacterial growth may consume oxygen and interfere with the calibration. If using the cup and you no longer have the sponge, place a small amount of clean water (1/8 inch) in the plastic storage cup instead.
3. Make sure there are no water droplets on the DO membrane or temperature sensor.
4. Then install the storage cup over the sensors. If using the cup, screw it on the cable and then disengage one or two threads to ensure atmospheric venting. Make sure the DO and temperature sensors are not immersed in water.
5. Turn the instrument on and wait approximately 5 to 15 minutes for the storage container to become completely saturated and to allow the sensors to stabilize.
6. Press Cal. Highlight Probe ID or User ID. User ID will appear automatically. Select 'None' if

you do not want a User ID stored with the calibration.
7. After selecting the User ID and/or Probe ID if appropriate, highlight DO % and press enter to confirm.
The instrument will use the internal barometer during calibration and will display this value in brackets at the top of the display. Highlight Barometer and press enter to adjust it if needed. If the barometer reading is incorrect, it is recommended that you calibrate the barometer.
8. Wait for the temperature and DO% values under “Actual Readings” to stabilize, then highlight Accept Calibration and press enter to calibrate.
9. The message line at the bottom of the screen will display “Calibrating Channel...” and then “Saving Configuration...”.

Protocol 2: tissue dissection for microbiome analysis. Protocol for downstream microbiome analysis and bioinformatics of the whole-clam microbial population and brood count tally.

- Sample clams from pond, retain those over 5 mm in shell length (measured adductor to adductor)
- If total brood size needs to be measured, collect clams into
    - separate PVC pipe with mesh held on each end by zip tie cages
    - individual 15 or 50 ml tubes if clams will be removed in a few hours
- If total brood size does not need to be measured, clams can be collected into a 15 or 50 mL tube, a Nalgene bottle, a cage, etc.
- Transport back in these individual containers with own pond water to lab
    - Place cages in ziplock of pond water. Do not mix ponds- keep the clams from lake A in bag of lake A water, to retain microbiome integrity.
- Sample a Nalgene of pond water sans clams for dissection

- Set up lab: Pour out rinse bins (10% bleach and DI H_2O) for any tool rinsing needed, get sterile 0.5 ml clam storage tubes, rack for clam tubes
- Clean bench, microscope, with bleach and RNAseaway. Wear gloves to avoid contamination of area
- Bleach clam tube rack in 10% bleach solution for at least 10 minutes prior to starting
- To dissect, for each clam you will need:
    - Autoclaved ultra fine forceps
    - Bleached (between each clam) plastic forceps
    - Sterile petri dish or lid
    - Autoclaved 2mL centrifuge tube for rinsing
    - Sterile scalpel blades (optional)
    - Kimwipes
    - **Cleaned** (between each clam) calipers
- Have 10% bleach soln prepared in the bin to sterilize the plastic forceps between uses (use one set> put in bin while you use your other set on the next clam> trade out sets>repeat)
    - Do not mix any tools or liquids used between clams

- Close and label sterilized 0.5 ml tubes for the clams. Weigh them, record in book
  Pour 50ml tube (sterile) of DI H_2O from autoclaved bottle, label
  Pour pond H_2O into 15ml tube, label
  Arrange bench so you work left to right- clam to bench, clam rinse, dissect

- Dissection:
  Spray gloves
- Using the blue plastic forceps, carefully get clam out of the bottle or other sampling container and into the 2mL rinse tube. **Retain the container- count extruded brood after dissection.**
- Pour in enough DIH_2O from the tube you poured before to suspend the clam
  Close, invert 3 times, open, pour off water but keep clam inside. Repeat 2x (clam gets rinsed 3 times total.)
    - Almost all clams will release detritus here. Some clams have lots of detritus on their shells. This will come off mainly in the first rinse. Do not rinse more than 3 times.
- Pour out wastewater

- Empty out clam onto clean Kimwipe
- Measure shell end to shell end lengthwise (adductor to adductor) with calipers, record shell length in mm
- Immediately transfer to petri dish on the dissecting scope using blue plastic forceps
  Re-spray gloves with RNAseaway
  Turn on microscope light
- Get clam into view under scope
- Hold clam gently with plastic forceps edge to edge (not across the wide part of the shell, it will break) so the hinge is on the dish.
- While gently holding clam in place, insert tip of fine tip forceps between shells and start gently working down the edges to break the seal and open the clam shells
- Ideally clam shell will butterfly open, if not the pieces of shell will have to be individually broken and removed
- Start looking for the brood inside the sacs/nestled in the tissue. If the sacs are visible/whole (unlikely), try not to tear or break them for ease of brood identification
- If possible, start removing the brood from the clam (look like small adults.)
    - Put them on the side of the dish. If not possible go to next step
    - They tend to be around 1mm in length
- Using forceps (and blade if needed) remove the whole clam body from the shell (scoop up the clam as one big blob with your forceps.)
    - Move shell to far side of dish. Add pond water to clam here if needed (from the tube poured before)
- Flatten out the clam body to get all shelled brood out of the whole body tissue blob.
    - You may be able to identify the 2 brood sacs surrounding the larval brood (miniscule clams) but often can't.
    - Larvae should be less than 1mm in length
    - Poke around in the tissue until you have a pile of larvae on one side of the dish and the examined tissue on the other.
- This process should go quick- 3 minutes. Count the brood *after* the body is safely in the tube.
- Have your labeled, pre-weighed clam tube ready. Put in whole clam body (not the brood or shell, they stay on the dish.)
    - Close tube. Weigh and record
    - Short term storage: lab freezer
    - Long term storage: -80
- Count the number of brood on your dish that were pulled out of the clam. Record
    - Larvae are miniscule and found inside the tissue/brood pouches
    - Shelled juvenile are the ones you found inside the mantle cavity
    - The number of extruded juveniles, if any, found inside the individual sampling container

- IF DOING ANOTHER STERILE CLAM:
- Change gloves
- Get new autoclaved fine tip forceps, bleach the blue plastic forceps, get new sterile petri dish or lid, new autoclaved 2mL centrifuge tube, clean Kimwipe, clean down the bench processing area where you handled the clam from the container>cleaning before dissection.
- RNAseaway gloves before getting the next clam sample from the bag

- IF MAKING A DISSECTION CONTROL:

- Follow all of the tool and bench area prep steps in the same way
- Skip the clam rinse steps
- Dip the autoclaved fine tip forceps in the sterile 2mL rinse tube of sterile DIH_2O, wipe liquid and forceps on the sterile petri dish on your scope
- Scrape around gently in the dish using both the autoclaved fine tips and the blue plastic forceps- try to replicate the motions you made doing dissection
- After 1 minute, wipe the forceps into the sterile labeled 1.5mL dissection control tube including any liquid drops remaining. (you'll extract this later, the tube will look empty so be careful not to lose it)
- Prepare for another clam dissection. Stagger your dissection controls between clam samples (stagger number depends on your samples.)

Protocol 3: non-microbial (non-sterile) clam dissection.

Protocol for brood counts, tissue metals, gene expression, protein expression, or biochemistry. Additional steps not reflected here (e.g., flash-freezing in liquid N2, sterilization steps with bleach and RNAse away) that may be relevant for gene or protein expression are not listed.

- Sample clams from pond, retain those over 5mm in shell length (measured adductor to adductor)
- If total brood size needs to be measured, collect clams into
    - separate PVC pipe with mesh held on each end by zip tie cages
    - individual 15 or 50 ml tubes if clams will be removed in a few hours
- If total brood size does not need to be measured, clams can be collected into a 15 or 50mL tube, a Nalgene, a cage, etc.
- Transport back in these individual containers with own pond water to lab
    - Place cages in ziplock of pond water
- Sample a Nalgene of pond water sans clams for dissection

- Set up lab: Pour out rinse bins (10% bleach and DI H_2O) for any tool rinsing needed, get sterile 0.5ml clam storage tubes, rack for clam tubes
- To dissect, get ultra fine forceps, a clean 15ml tube for rinsing, 15ml tube for pond water, clean petri dish or lid to dissect in, possibly scalpel, paper towel for draining, and clean plastic forceps.
    - Mixing between clams is okay as long as the tools are clean (no debris/tissue between clams)- rinse in bin if you get detritus

- Wear gloves. Keep areas clean to avoid contaminating clams with environmental metals or introducing anything that could degrade the RNA, if that's what your end goal is
- Clean bench and dissecting scope according to cleanliness procedures for your target (RNA, metals, etc)
- Make sure plastic forceps are clean
- Get wastewater container for clam rinse water
- Close and label sterilized 0.5ml tubes for the clams. Weigh them, record in book
  Pour 50ml tube (sterile) of DI H_2O from autoclaved bottle, label
  Pour pond H_2O into 15ml tube, label
  Arrange bench so you work left to right- clam to bench, clam rinse, dissect

- Dissection:
- Using spoon, carefully get clam out of bottle or other sampling container and into the 15ml rinse tube. **Retain the container- count extruded brood after dissection.**
- Pour in enough DIH_2O form the tube you poured before to suspend the clam
  Close, invert 3 times, open, pour off water but keep clam inside. Repeat 2x (clam gets rinsed 3 times total.)
    - Almost all clams will release detritus here. Some clams have lots of detritus on their shells. This will come off mainly in the first rinse. Do not rinse more than 3 times.
- Pour out wastewater
- Empty out clam onto paper towel
- Measure end to end with calipers, record shell length in mm
- Immediately transfer to petri dish on the dissecting scope using plastic forceps
  Turn on microscope light

- Get clam into view under scope
- Hold clam gently with plastic forceps edge to edge (not across the wide part of the shell, it will break) so the hinge is on the dish.
- While gently holding clam in place, insert tip of fine tip forceps between shells and start gently working down the edges to break the seal and open the clam shells
- Ideally clam shell will butterfly open, if not the pieces of shell will have to be individually broken and removed
- Start looking for the brood inside the sacs/nestled in the tissue. If the sacs are visible/whole (unlikely), try not to tear or break them for ease of brood identification
- If possible, start removing the brood from the clam (look like small adults.)
    - Put them on the side of the dish. If not possible go to next step
    - They tend to be around 1mm in length
- Using forceps (and blade if needed) remove the whole clam body from the shell (scoop up the clam as one big blob with your forceps.)
    - Move shell to far side of dish. Add pond water to clam here if needed (from the tube poured before)
- Flatten out the clam body to get all shelled brood out of the whole body tissue blob.
    - You may be able to identify the 2 brood sacs surrounding the larval brood (miniscule clams) but often can't.
    - Larvae should be less than 1mm in length
    - Poke around in the tissue until you have a pile of larvae on one side of the dish and the examined tissue on the other.
- This process should go quick- 3 minutes. Count the brood *after* the body is safely in the tube.
- Have your labeled, pre-weighed clam tube ready. Put in whole clam body (not the brood or shell, they stay on the dish.)
    - Close tube. Weigh and record
    - Short term storage: lab freezer
    - Long term storage: -80
- Count the number of brood on your dish that were pulled out of the clam. Record
    - Larvae are miniscule and found inside the tissue/brood pouches
    - Shelled juvenile are the ones you found inside the mantle cavity
    - The number of extruded juveniles, if any, found inside the individual sampling container
- IF DOING ANOTHER NON-STERILE CLAM:
- Clean the forceps, plastic forceps, petri dish, scalpel- everything used for the dissection besides the paper towel. Shell fragments can stick to the forceps easily.
- Get a new paper towel for draining

- If you need pooled samples, repeat this process 3 times but place the clam tissues into 1 tube.
    - Record all length and brood information for each individual

DNA extraction protocol:

Note: after the extraction had been completed, we learned that some steps could have been optimized. This protocol is therefore a documentation of what was done and should not be followed without alteration in the future.

Supply list:

Lysis buffer: prepared by and in the Walke lab as follows:

• Prepare and autoclave stock solutions of
-Tris-HCl pH 8 1M (calibrate pH with HCl)
-EDTA pH8 0.5M (calibrate pH with NaOH pellets)

• Prepare and autoclave lysis buffer
-20mM Tris-HCl pH 8
-2mM EDTA pH 8
-1.2% Triton-x-100

Extraction:
P200 + filter tips
P1000 + filter tips
Tip waste
Sterile pestles: autoclaved pre-use
Sterile swabs (single use wood and cotton swabs in sterile packets)
15 mL tube of autoclaved DI water
Sterile tube of autoclaved molecular grade water
2mL sterile tubes
1.5mL sterile tubes- pre-label
Tube racks (1 to hold 50mL tubes, 1-2 to hold DNA extraction tubes depending on number of samples extracted)
Dneasy Blood and Tissue Kit (including the Proteinase-K that is stored in the fridge)
100% cold ethanol (stored in freezer until use, should be in 50 mL tube)
Fine-tip Sharpie

Lysis buffer:
Lysozyme (from Walke lab freezer)
Lysis buffer (from Walke lab) that lysozyme will be mixed into, usually stored in 50mL tube
Sterile spatula
Calculator
Sterile pouring-off buffer 50mL tube
P1000 + tips
Notebook
Pen

Buffer preparation: same for clam and water samples

1. Set heat block to 37°C. Fill spaces 3/4 with DI water; confirm temperature of multiple spaces.
2. If extracting DNA from water samples: Make sure water samples are thawed
   Water samples that were frozen at -80 must be thawed in fridge for 36 hours for 45 mL samples.
   Thawing at room temperature could cause microbial growth

3. Make sure bench is clean
4. Clean and set out:
   P200 + filter tips
   P1000 + filter tips
   Tip waste
   Sterile pestles: autoclaved pre-use
   Sterile swabs (single use wood and cotton swabs in sterile packets)
   15 mL tube of autoclaved DI water
   Sterile tube of autoclaved molecular grade water
   2mL sterile tubes
   1.5mL sterile tubes- pre-label
   Bleached racks
   -Racks bleached in 10% bleach and DI water soln for 15 minutes before use
5. Locate Dneasy Blood and Tissue kit; ensure all reagents are present and mixed with ethanol if needed as per the manufacturer's directions
6. Do calculations for lysis buffer:
   Conversion factor for lysis buffer: 48 mg lysozyme + 2.4 mL lysis buffer = 12 samples (48 mg/ 2400 µl)
   Create enough lysis buffer for **([n] samples + 1) x 180 µl**
   Example calculation: 5+1 samples x 180 µl = 1080 µl
   (48 mg/ 2400 µl) = (X mg/ 1080 µl)
   2400 (X) = 1080 (48) **cross multiply, divide to isolate X**
   x = 21.6 mg (lysosome that you will put into your empty tube, then add 1080 µl lysis buffer)
7. **Supply list for Walke lab: mix lysis buffer in Walke lab**
   Sterile spatula
   Calculator
   Sterile 50 mL tube (3) for buffer + lysozyme
   Matos lab stock buffer tube (1)
   Sterile pouring-off buffer 50 mL tube (2)
   P1000 + tips
8. Pipette buffer from stock tube (1) to holding tube (2)
   Get tube holder for tube on scale from drawer, set buffer tube (3) on scale and tare
   Get lysozyme from freezer
   Using sterile spatula and aseptic technique, measure out pre-calculated amount of lysozyme (the mg calculated in step 6) into tube 3 on scale. Be careful as you are pouring dry lysosome into empty tube and can spill easily. Close tube.
   Your mg will probably be slightly different than calculated. Put lysozyme away, and adjust math:
   ([mg lysozyme in tube]/ X µl) = (48 mg/ 2400 µl)
   [mg lysozyme in tube](2400 µl)/48 mg = **µl of buffer that you pipette from tube 2 into the tube containing the lysozyme (tube 3)**
9. Once you have added the buffer to the lysozyme, close and invert tube, vortex briefly. Will be bubbly.
   Mixed lysis buffer does not keep- discard buffer if not used within 12 hours of mixing.

10. If processing tissues, get samples from -80°C freezer on way back to lab/in lab. Should be ready to start as soon as possible after mixing the buffer.
    EXTRACTION: CLAM
11. Add 180 µl of buffer + lysozyme to each pre-labeled 1.5 mL tube (extraction controls and clam containing)
    Close all tubes.
12. Grind clam tissue in lysis solution with sterile pestle for 5 seconds to homogenize. Use new pestle for each sample. Keep used pestles to rinse and re-autoclave after.
13. Dissection and extraction controls: nothing further is done after adding buffer, just close the tube and process like the others
    EXTRACTION: WATER
14. Water samples: invert thawed water sample a few times
15. Have the sample tubes filled with buffer before sampling the water tube
16. Using sterile swab, sample 8-10 cm down in water tube
17. Swirl swab in water for 7 seconds
18. Swirl this same swab in the buffer-filled water sample tube for 5 seconds
19. Water sample control:
20. Repeat steps 16-18 using sterile tube of autoclaved DI H20 instead of water sample

    ALL SAMPLES:

21. Incubate all tubes in 37°C heat block for 1 hr, rotating position every few minutes. Inserting tubes in water in heat block may cause slight overflow.
22. Check Dneasy kit components again:
    Each tube will get 25 µl Proteinase K; this is from the Dneasy kit but is kept in the fridge.
23. Turn on other heat block to 56°C, fill with water
24. Get out buffer AL from Dneasy kit.
25. After samples finish their 1hr incubation, to each tube add:
    25 µl proteinase k
    200 µl buffer AL (be very careful, buffer is “slippery”, use aseptic technique)
26. Vortex each sample.
    Incubate at 56°C for 30 minutes
27. While incubating, set up Dneasy kit filter/collection tubes and sterile 1.5 mL DNA storage tubes
    Each sample will have a filter+ collection tube, 2 collection tubes, 1 1.5 mL sterile tube for 37-41, and 1 final 1.5 mL sterile storage tube.
28. Get out 100% cold ethanol from freezer right before tubes come out of block
29. When tubes are finished incubating, add
    200 µl cold ethanol to each tube
    Vortex 5-10 seconds
30. Using P1000 set to 800 µl, pipette ALL contents of tubes into their respective filter + collection tubes.
31. Centrifuge at 8,500 rpm 1 minute at room temperature
32. Discard the liquid in the collection tube along with the tube. Retain the filter, place into new collection tube.
33. Add 500 µl AW1 buffer to each collection tube (very careful, aseptic technique)
34. Centrifuge at 8,500 rpm 1 minute at room temperature

35. Discard liquid and tube. Place the filter into new collection tube
36. Add 500 µl AW2 buffer to each tube (very careful, aseptic technique)
37. Centrifuge 14,000 rpm 3 minutes at room temperature
    Discard liquid (retain tube)
    Centrifuge 1 minute
    Discard liquid and tube, be careful not to splash on filter
38. Place each filter into their respective 1.5 mL storage tubes
39. Add 100 µl sterile MGW directly onto center of membrane in each tube
40. Incubate at room temp for 5 minutes
41. Centrifuge at 8,500 rpm for 1 minute at room temperature
    Position vials so caps don't break- bend outer cap back over the rim of centrifuge
42. Pass the 100 that was spun down back through the filter
    Incubate again for 5 minutes
    Centrifuge 8, 500 rpm for 1 minute
43. Remove filters, pipette the 100 ul of eluted DNA into sterile 1.5 mL storage tube
44. You're done! Quantify using Qubit* before any freeze/thaw cycle
45. Store at 4°C if using within a month, store at -80°C long term.

*Follow Qubit protocols for your appropriate kit and concentrations

Protocol 4: Illumina MiSeq Sequencing Protocol

Adapted from the Earth Microbiome Project
Edited in August, 2017 by: Jeni Walke, Angie Estrada, Daniel Medina, Jessica Hernandez and Lisa Belden. Edited Nov-April 2018-2019 by: Shelby Fettig, Jeni Walke

Reagents:
UltraClean PCR grade H2O

5 Prime Hot Master Mix

Forward primer + barcode IL 515F

Reverse primer IL 926R

Before beginning:

- Sterilize workspace with RNA away. If possible, perform in a hood dedicated to PCR set up. UV hood before using; UV hood space 15 minutes and open PCR tubes for additional 15 minutes.
- Sterilize pipettors (**use pipettors dedicated for PCR** reagents and use a separate pipettor for the DNA) with bleach and ethanol or with RNA away.
- Clean and sterilize with 5% bleach: 1 large centrifuge tube rack and several small PCR tube racks. Rinse and allow to dry.
- Locate samples and barcodes. Assign samples to barcodes. Keep both in fridge until ready to use.

Step 1: Make your PCR reactions

A) For each sample, you will run triplicate PCR reactions plus a negative control = 4 PCR tubes per sample.
B) For samples that might have LOW DNA CONCENTRATIONS, the PCR reactions could be prepared with the same method as below, but with a small change in the volume of the reagents and DNA; additionally, BSA could be added to increase PCR yield.

Per sample	4x (4 per sample – triplicate + neg control)
12 ul UltraClean PCR grade H2O	48 ul
10 ul 5 Prime Hot Master Mix	40 ul
0.5 ul Forward primer + barcode IL 515	2 ul
0.5 ul Reverse primer IL 926R	2 ul

23 ul Total (Before DNA)

2.0 ul DNA 6.0 ul (in triplicate) = 25 ul rxn

1. Set up four rows of PCR tubes. The front row is where we will set up our M^3. The second row will be our negative control. The last two rows will be our second and third run of samples.

2. Add all reagents, **except DNA**, to each PCR tube in the first row of the plate.
    a. Each tube will have its own forward primer with assigned barcode; add forward primer last to avoid changing tips between each tube for all other reagents.
    b. Label each tube by barcode.
    c. Vortex gently and briefly centrifuge after all reagents except DNA have been added to all tubes in front row.

3. Pipette 23 ul from the first row of PCR tubes, with every reagent listed above except DNA, into the negative PCR tubes. Label each tube as negative control + barcode number.

4. Add DNA (6ul) to first row.
    a. Vortex gently and centrifuge briefly

5. Take 25 ul from the first row of PCR tubes and add into replicate rows #2 and #3. Label tubes in second and third row with barcode #.

6. Vortex gently and centrifuge each PCR tube, including negative control strip, briefly.

Step 2: Run reactions in thermocycler

1. Make sure machine is set for 25 ul samples.

2. Thermocycler conditions:

Temp	Time	
1. 94°C	3 min	
2. 94°C	45 sec	Denaturing
3. 50°C	1 min	Annealing
4. 72°C	1.5 min	Extension
○ Repeat steps 2-4 34x		
5. 72°C	10 min	
6. 4°C	hold	

You can maintain your PCR product in the fridge overnight if you need to wait until the next day to run your gel.

Step 3. Run gels to check amplification and negative controls

1. Combine your three separate PCR reactions into a single PCR tube. Use post-PCR pipettors and tips. Use new pipet tip for each sample.

2. Make a 1.5% gel. Combine 1X TBE and agarose in a small Erlenmeyer flask. Microwave until just boiling. Swirl. Continue boiling/swirling until solution is clear.

a. Mini-gels:

i. 1% 40 mL buffer, 0.4 g agarose

ii. 1.5% 40 mL buffer, 0.6 g agarose

b. Big gels:

i. 1% 140 mL buffer, 1.4 g agarose

ii. 1.5% 140 mL buffer, 2.1 g agarose

3. Once the solution has cooled slightly, add gel red stain (or GreenGlo).

a. Mini-gels: 4 ul (0.4ul for GreenGlo)

b. Big gels: 14 ul

Note: Gel red is the dye that stains your DNA for visualization.

Note: Gel red stain is light sensitive--keep away from light as much as possible.

4. Pour gel into mold and allow to cool completely. Don't forget the combs!

5. On a strip of parafilm, combine 5 ul PCR product and 1 ul loading dye. Pipette up and down to combine.

Note: loading dye is the dye that is used to view how far your samples have traveled in the gel during electrophoresis.

6. Pipette each sample and negative control into gel well (5ul of PCR product and dye combined.).

As the amount of solution decreases (due to evaporation), you may need to reset your pipette ul setting. Avoid air bubbles in the pipette tip as this will cause the DNA to leak out. Gently pipette solution into wells.

7. Load 5 ul of DNA ladder into gel (one lane per row). You can use a broad range 50-10,000 bp ladder.

8. Run gel at a voltage of ~160V for approximately 20 minutes, until dye is about halfway across gel and each of the three colored bands has separated. Longer time for larger DNA fragments, larger gels.

9. Visualize gels using ImageLab software. Do not touch the computer, gel imager, or handle on gel tray with gloved-hands to avoid getting sticky buffer on equipment. Bands for this primer set will be at ~ 300-350 bp. Sample bands may be a little smeary, but there should not be multiple bands. No bands should be visible for the negative controls.

NOTE: If sample bands are very faint (indicating too low or too high DNA content), try the following alternatives (see table):

a) Modify the starting DNA concentration with 1:10 or 1:50 dilutions. Or use ½ of the DNA volume. Dilute in PCR water.

b) Reduce de volume of water (for example: 4ml/sample) and replace with BSA which increases PCR yield (also useful when bands are not amplifying).

c) If the previous does not work, is possible that DNA is too low in which case duplicate the volume of DNA samples (to 4ml) or try to duplicate DNA + BSA

	Original Reaction		BSA only		½ DNA		2XDNA + BSA	
	Per sample	4X Vol	Per sample	4X Vol	Per sample	4X Vol	Per sample	4X Vol
PCR grade H20	12	48	10	40	13	52	9	36
5Prime Hot MasterMix	10	40	10	40	10	40	10	40
Forward IL 515F	0.5	2	0.5	2	0.5	2	0.5	2
Reverse barcode	0.5	2	0.5	2	0.5	2	0.5	2
DNA (3x)	2	6	2	6	1	3	4	12
BSA			2	8			1	4
Total	25	98	25	98	25	98	25	98

NOTE: If there are bands in the negative control for a sample, redo the PCR.

Store PCR products at -20 C until you've accumulated all of the samples that you are going to run on a single Illumina plate before moving on to Step 4.

Step 4: Quantifying the DNA

We use a Qubit 4.0 Fluorometer and the dsDNA High Sensitivity assay kit. Readings can be a bit fickle, so it is better to do all of your samples on the same day at the same time with the same working solution and standards. This can be done on the countertop. Use post-PCR pipettors and tips.

Before beginning:

- Organize your samples in a single PCR tube rack on ice.
- Label fluorometry tubes supplied by Qubit in a tube rack with sample names, in the same order as they occur in the PCR tube rack.

1. Add appropriate volume of Qubit working solution into a 50ml centrifuge tube (enough for samples and 2 standards).

2. Make your standards. Combine 10 ul of each standard with 190 ul working solution. Make a separate solution for each standard and combine in the tubes supplied by Qubit.

3. For your samples: Combine 2-5 ul sample with 198-195 ul working solution. Total solution volume should be 200 ul. Make a separate solution for each sample and combine in the fluorometry tubes that you labeled already. To get the most accurate measurements, it is very important that you get the precise amount of your entire sample into the working solution. Try 2 ul of sample first. If the readings are too low (there's too little DNA), then redo, increasing the amount sample.

4. Vortex and briefly centrifuge all tubes. Drops of liquid stuck on the sides or lids of tubes can mess up the readings.

5. Incubate at room temperature for 2 min.

6. Read tubes in the Qubit Fluorometer. Specify the amount of sample you used (i.e., 2-5 ul). Record reading in ng/ul.

Step 5: Combine equal amounts of amplicons into a single tube
1. Based on the concentration determined by the Flourometer, determine how much of each sample you need to add. The goal is to add the same amount of ng of DNA per sample (~180-200 ng) into a single, 1.5 ml centrifuge tube.
Example: If Sample 1 has a concentration of 38 ng/ul, you should add 200/38 = 5.3 ul to the pool.

2. Add the appropriate volume of each sample to a single low-retention 1.5ml centrifuge tube using post-PCR pipets. This is your pooled sample. Keep on ice. Compute the total volume of the pooled sample before adding to make sure the volume will fit in a 1.5ml tube.

Step 6: Clean up pooled sample.
We use the Qiagen QIAquick PCR Clean Up Kit.
If this is the first time you are using the kit, make sure you add ethanol and the PH indicator to the appropriate buffers as described in the manufacturer's instructions.

1. Vortex the pooled sample to thoroughly mix it. Pipette 100 ul of the pooled sampled into a new, clean low-retention 1.5 ml centrifuge tube. **Store the remaining, uncleaned pooled sample in storage box in -20C with other uncleaned pools.

2. Add 500 ul of Buffer PB to the 100 ul of your pooled sample. Vortex. Check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.

4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s at 13,000 rpm.

5. Discard flow-through. Place the QIAquick column back into the same tube.

6. Wash the pooled sample. Add 0.75 ml Buffer PE to the QIAquick column, let the buffer sit on the filter for 2 min, then centrifuge for 30–60 s at 13,000 rpm.

7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at 13,000 rpm.

8. Place the QIAquick column in a new, clean 1.5 ml centrifuge tube.

9. To elute the DNA, add 50 ul water to the QIAquick column, let the buffer sit on the filter for 3 min, then centrifuge for 1 min at 13,000 rpm.

10. Measure the concentration of the cleaned, pooled sample using the Qubit Fluorometer (as above, but with only one sample, still need the 2 standards), and the 260/280 ratio using the Nanodrop should be between 1.8-2.0.

Step 7: Add PhiX
***Latest run (Jan 2019) sequencing facility added PhiX for us
For running these libraries in the MISeq and HiSeq, you may need to make your sample more complex by adding 30-50% PhiX to your run.

However, the sequencing facility may add PhiX for you. Check with the particular sequencing facility you are using for information about adding PhiX. The sequencing facility that we use (listed below) adds PhiX for you.

Step 8: Send for sequencing!
Keep cleaned, pooled sample frozen until ready to send. Send sample on dry ice.

Sequencing Facility and contact info:

Maura Berkeley and
Zach Herbert <zherbert@research.dfci.harvard.edu>
Molecular Biology Core Facilities
Dana Farber Cancer Institute at Harvard
http://mbcf.dfci.harvard.edu/

Protocol 5: QIIME2 Bioinformatics Processing Steps

Install QIIME2: I ran it using VirtualBox for Windows, following the installation process listed on their website. https://docs.qiime2.org/2019.10/install/virtual/virtualbox/

The sequencing files I received were already demultiplexed (one file per sample sequenced). All files used were single end reads.

Import data:

Making manifest file:

The manifest file is used to import the sequences into QIIME2. It has two columns: "sample-id" with the id used in the mapping file, and "absolute-filepath" with the exact location of the file on your computer. The manifest file must be saved in the same folder as the fasta files.

To create the manifest file, I made a worksheet in GoogleSheets as described above, listing the sample ID that matched the ID from the mapping file and the absolute filepath of the fasta files that I had already imported into QIIME2. I then validated the data using the GoogleSheets extension Keemei, downloaded the file named "Manifest.txt" as a .tsv, and transferred it to the same folder that my fasta files were in.

Import and visualization:

As the exact code used will vary depending on your installation, your filenames, and your data, I am showing the general plugins used in the general sequence they should be used in.

Use this code for a single end read and when importing demultiplexed files:

```
qiime tools import \
--type 'SampleData[SequencesWithQuality]' \
--input-path Manifest.txt \
--output-path single-end-demux.qza \
--input-format SingleEndFastqManifestPhred33V2
```

Summarize sequencing data and view quality scores

```
qiime demux summarize \
--i-data single-end-demux.qza \
--o-visualization single-end-demux.qzv
```

I ran deblur (there are other filtering options). The process took 22 hours.

Quality control using Deblur Filter by quality score:

```
qiime quality-filter q-score \
--i-demux single-end-demux.qza \
--o-filtered-sequences demux-filtered.qza \
--o-filter-stats demux-filter-stats.qza
```

Then complete deblur process:

```
qiime deblur denoise-16S \
```

--i-demultiplexed-seqs demux-filtered.qza \
--p-trim-length 250 \
--o-representative-sequences rep-seqs-deblur.qza \
--o-table table-deblur.qza \
--p-sample-stats \
--o-stats deblur-stats.qza

Dr. Walke assigned taxonomy, filtered mitochondria and chloroplasts using a pre-trained feature classifier the computer that I had access to lacked the RAM necessary for this step. I have included the outline of the code I would have run. It does not include training a feature classifier, which may be necessary for some QIIME2 processes.

Taxonomy:

qiime feature-classifier classify-sklearn \
--i-classifier silva-132-515-926-nb-classifier-PC.qza \
--i-reads rep-seqs-deblur.qza \
--o-classification taxonomy.qza

Filter Mitochondria and Chloroplasts from Table:

qiime taxa filter-table \
--i-table table-deblur.qza \
--i-taxonomy taxonomy.qza \
--p-exclude D_4__Mitochondria,D_3__Chloroplasts,D_3__Chloroplast,Unassigned \
--o-filtered-table filtered-table.qza

qiime feature-table summarize \
--i-table filtered-table.qza \
--o-visualization filtered-table.qzv \
--m-sample-metadata-file sample-metadata.tsv

Filter Mitochondria and Chloroplasts from Sequences:

qiime taxa filter-seqs \
--i-sequences rep-seqs-deblur.qza \
--i-taxonomy taxonomy.qza \
--p-exclude D_4__Mitochondria,D_3__Chloroplasts,D_3__Chloroplast,Unassigned \
--o-filtered-sequences filtered-rep-seqs.qza

Visualize Taxonomy:

qiime metadata tabulate \
--m-input-file taxonomy.qza \
--o-visualization taxonomy.qzv

At this point, the 56 samples have been filtered and assigned taxonomy.

I filtered the features that were present in my sequenced DNA extraction control sample out of the other 55 samples. I followed the documentation for the plugin available at: https://docs.qiime2.org/2020.2/plugins/available/feature-table/filter-samples/

Filter control sample out of table

qiime feature-table filter-samples \
--i-table FeatureTable.qza \
--m-metadata-file sample-metadata.tsv
--p-where "\"#SampleID\" NOT IN ('CL')" \
--o-filtered-table filtered_table.qza

I then filtered these same features out of the sequences (the previous step only filtered them from the table). I followed the documentation for the plugin available at: https://docs.qiime2.org/2021.4/plugins/available/feature-table/filter-seqs/

Filter control sample out of sequences

qiime feature-table filter-seqs \
--i-data FeatureData.qza \
--i-table filtered_table.qza \
--p-where "\"#SampleID\" NOT IN ('CL')" \
--o-filtered-data filtered_seqs.qza

I used these "filtered_table" and "filtered_seqs" for the rest of the analyses.

Visualize Microbiome Using Taxa Bar Plots

qiime taxa barplot \
--i-table filtered-table.qza \
--i-taxonomy taxonomy.qza \
--m-metadata-file sample-metadata.tsv \
--o-visualization taxa-bar-plots.qzv

Generate a tree for phylogenetic diversity analysis

qiime phylogeny align-to-tree-mafft-fasttree \
--i-sequences filtered-rep-seqs.qza \
--o-alignment aligned-rep-seqs.qza \
--o-masked-alignment masked-aligned-rep-seqs.qza \
--o-tree unrooted-tree.qza \
--o-rooted-tree rooted-tree.qza

Alpha Rarefaction Plotting

qiime diversity alpha-rarefaction \
--i-table filtered-table.qza \
--i-phylogeny rooted-tree.qza \
--p-max-depth 23899 \

--m-metadata-file sample-metadata.tsv \
--o-visualization alpha-rarefaction.qzv

Alpha and Beta Diversity (Core Metrics)

qiime diversity core-metrics-phylogenetic \
--i-phylogeny rooted-tree.qza \
--i-table filtered-table.qza \
--p-sampling-depth 5312 \
--m-metadata-file sample-metadata.tsv \
--o-rarefied-table rarefied-table.qza \
--output-dir core-metrics-results

Output folder will contain alpha metrics (Default: Shannon, observed otus, faith's, evenness) and beta metrics (jaccard, bray-curtis, unweighted unifrac, weighted unifrac) all at the same time.

Alpha diversity stats: Kruskal-Wallis

1. Shannon

qiime diversity alpha-group-significance \
--i-alpha-diversity core-metrics-results/shannon_vector.qza \
--m-metadata-file sample-metadata.tsv \
--o-visualization core-metrics-results/shannon_significance.qzv

Repeat for Observed OTUs, Faith's Phylogenetic Diversity, and Evenness.
I suggest changing the output name so you avoid accidentally replacing files if you redo a measure.

Alpha diversity stats: Correlation

1. Shannon
qiime diversity alpha-correlation \
--i-alpha-diversity core-metrics-results/shannon_vector.qza \
--m-metadata-file sample-metadata.tsv \
--o-visualization core-metrics-results/shannon_correlation_Spearman.qzv
Repeat for Observed OTUs, Faith's Phylogenetic Diversity, and Evenness.

Beta diversity stats: PERMANOVA

1. Bray-Curtis

qiime diversity beta-group-significance \
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \
--m-metadata-file sample-metadata.tsv \
--m-metadata-column ChytridResult \
--o-visualization core-metrics-results/bray_curtis_chytridresult_significance.qzv \
--p-pairwise

Repeat for Jaccard, Unweighted UniFrac, and Weighted UniFrac.

CURRICULUM VITAE

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Education

2019-2021	**Eastern Washington University** M.S. Biology June 2021 (expected)
2014-2019	**Eastern Washington University** B.S. Biology June 2019 B.A. Psychology June 2019

Awards

2021	EWU Department of Biology Outstanding Graduate Student
2020-2021	EWU Biology J. Herman, Jean Swartz, & Nate Narrance Graduate Fellowship
2020-2021	Pride Foundation Scholarship
2019-2021	Graduate Student Assistantship, Department of Biology, Eastern Washington University
2014-2019	Leadership 1000 Scholar, College Success Foundation
2014-2015	Presidential Scholarship, Eastern Washington University

Employment

2019-2021	**Eastern Washington University Biology department, Teaching Assistant** Preparing and managing undergraduate labs, aiding teachers in and out of class, grading coursework, assisting undergraduates in group and individual settings
2018-2019	**Eastern Washington University Biology department, Research Assistant** Wetland sampling, data management and analysis
2017-2019	**Spokane, Washington, various community theater groups, stagehand** Managed and built props, stage equipment Mediated interactions between audience and actors
2016-2018	**Residential Housing Office Aide, Eastern Washington University Housing** Provided information about safety, regulations, academic and social activities for undergraduates residing in campus housing Worked with EWU and Cheney emergency services to create safe student environments

2016	**Eastern Washington University PLUS tutoring program** Course: Critical Thinking (Phil 210) Taught 1-25 students in group or individual settings Participated in weekly tutor training sessions to learn new education techniques

Teaching Assistantships

2021	Molecular Biology (Biol 438), Animal Ecophysiology (Biol 490)
2020-present	Biology of Symbiosis (Biol 345)
2020	Biology I (Biol 171)
2019-present	Fundamentals of Genetics (Biol 310)
2019-present	Biological Investigation (Biol 270)

Volunteer experience

2020-present	**Biology Graduate Student Liaison to Department Meeting** Attended weekly Biology Department faculty meetings to help disseminate information between the faculty, staff, and graduate students
2020	**ENVISION by WiSTEM Proposal-writing Competition judge** Read, evaluated, and judged research proposals for nationwide science competition held by the Women In STEM initiative
2018-present	**Satori Summer Camp, Counselor in Training** Mentored and supervised youth grades 6-12 in overnight academic summer camp Aided instructors in classroom management, camp activities, and crisis intervention
2018-2019	**Eastern Washington University Biology Department, Lab Assistant** Dr. Joanna Joyner-Matos Lab Sampled and identified aquatic macroinvertebrates, plants Collected and managed macroinvertebrate data, generated preliminary summaries
2018-2019	**Eastern Washington University Psychology Department, Lab Assistant** Dr. Theresa Martin Lab Collected, managed, analyzed personal student data from experimental surveys Trained incoming undergraduate in statistical software and database cleanup
2017-2018	**Eastern Washington University Theater, Workshop Assistant** Built props for EWU theater plays Became familiar with a variety of woodworking and power tools, safety procedures
2016	**Eastern Washington University Biology Department, Lab Assistant** Dr. David Daberkow Lab Handled vivarium rats, administered behavioral assays

Collected and organized assay data

Community service

2006-present	**Community Cultural Engagement, Spokane Indian Reservation (primarily)** Performed culturally significant activities for community events: funerals, celebrations, ceremonies Transferred endangered cultural knowledge including drum songs, traditional food collection and storage, and some language skills
2016-present	**Science Outreach** Participated in biology community outreach programs and events: Walk for MS, Cheney Mayfest, Spooky Science Practiced science communication in a non-academic environment

Research presented

2021	“Metal-exposed clams exhibit different investment in somatic growth and reproduction”, Dr. J. Joyner-Matos. EWU Student Research and Creative Works Symposium
2020	"Metal-Exposed Freshwater Clams Exhibit Greater Investment in Somatic Growth Than in Reproduction", Dr. J. Joyner-Matos, Dr. C. Nezat. Society of Environmental Toxicology and Chemistry North America 41st Annual Meeting
2020	"Preliminary Microbiome Analysis of Freshwater Bivalves from Turnbull National Wildlife Refuge", Dr. J. Joyner-Matos. EWU Student Research and Creative Works Symposium
2019	“Effects of restoration techniques on wetland macroinvertebrate abundance and diversity in and around Turnbull National Wildlife Refuge”, Dr. J. Joyner-Matos. EWU Student Research and Creative Works Symposium
2019	“Impact of Conflict in Married v. Divorced Parents”, Dr. T.J. Martin. EWU Student Research and Creative Works Symposium

Clubs and Orgs

2020-present	**President, EWU Biology Graduate Student Organization**
2019-2020	**Vice President, EWU Biology Graduate Student Organization**
2016-2017	**Vice President, EWU Neuroscience Research Driven Students**

Relevant coursework:

Biol 301 (Microbiology, with lab)
Biol 303 (Invertebrate Zoology, with lab)
Biol 345 (Biology of Symbiosis, with lab)
Biol 353 (Microbial Physiology)
Biol 380 (Data Analysis for Biologists)

Biol 399 (Directed Study, Microbiome Multiple Sclerosis)
Biol 399 (Directed Study, Animal Ecology)
Biol 423 (Evolution)
Biol 436 (Cell Biology)
Biol 440 (Ecology)
Biol 490 (Senior Capstone: The Microbiome, with lab)
Biol 496 (Host-Pathogen Interactions)
Biol 512 (Current Topics in Physiology)
Biol 513 (Current Topics in Cell and Molecular Biology)
Biol 514 (Current Topics in Ecology and Evolution)
Biol 596 (Microbial Ecology, with lab)