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Hot and Salty: Assessing ecological stress in New Hampshire streams at community, population, and molecular levels

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Basic Information

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Hot and Salty: Assessing ecological stress in New Hampshire streams at community, population, and molecular levels

Problem

New Hampshire's climate is expected to resemble that of the US Mid-Atlantic by 2100 (USGCRP 2009). With this shift comes increased air temperatures, less snow pack, more ice storms, and more rain on snow events. From a freshwater ecology perspective, much of central and northern New Hampshire's streams are currently populated by coldwater species (e.g., Brook trout; Neils 2009). As a result of increasing air temperatures, stream temperature will likely increase; however, the increase will vary among streams (Kelleher et al. 2011). For many species, this thermal shift may be within their fundamental tolerance range (e.g., 21°C thermal maxima for Brook Trout), barring additional physiological stress. However, growth in development (e.g., roads, housing) and energy production (mining, fracking) in northeastern states is causing additional stress on freshwater biota (Van Meter et al. 2011, Kelting et al. 2012). Among emerging concerns are the short-term and cumulative impacts of thermal and salinity stress on freshwater resources and biota (Findlay and Kelley 2011, Cuffney et al. 2010, Van Meter et al. 2011, Dalinsky et al. 2014, Stitt et al. 2014).

Road deicers are an emerging concern in NH where a 'bare pavement' policy has been adopted (TRB, 1991). Applied salts are flushed from roadways in early spring and during mid-winter thaw events. Some are immediately incorporated into surface waters while others infiltrating into subsurface flow and groundwater before reaching streams (Daley et al. 2009). The movement of sodium chloride into soil and groundwater systems delays the emergence of salts in streams, resulting in elevated Cl concentrations into summer months (Williams et al. 2000, Findlay et al. 2011, Kelting et al. 2012), a sensitive time for growth, development, and reproduction of freshwater biota. The impacts of thermal variability and salt loading on freshwater biota have garnered attention and study in northern states, but it remains unclear how the synergy of salt and thermal stressors impact biota across the community, population and molecular levels.

Traditionally, biotic response to water quality degradation is measured using broad-based community metrics (e.g., Simpson's Index of Diversity) and/or assessing populations of select bio-indicators (e.g., EPT= the macroinvertebrate orders of Ephemeroptera, Plecoptera, and Tricoptera). More recently, researchers look to family and genus level abundance as indicators of water quality (Carlisle et al. 2008). However, both approaches are largely reliant on the loss of individuals and/or species, which could have cascading effects on biodiversity and the ecological function of streams. In order to avoid the potentially negative effects of osmo-thermal stress on NH stream biota, we need studies that investigate subtle biotic responses along a gradient of salt and thermal stress. Collectively, this means monitoring the overall composition of the benthic macroinvertebrate community as well as stress at the individual level via biomarkers.

Biomarkers are parameters serving as objective and quantifiable characteristics of biological processes. These can include indications of unintended environmental exposure (Strimbu & Tavel, 2010). A good biomarker is one that can be used to model dose-effect relationships for clinical diagnoses and monitoring purposes (Van Der Oost et al. 2003). We sought a biomarker capable of representing the sublethal stress response to NaCl. Harmful effects of chemical introduction into the environment may not be readily apparent; some deleterious effects at the organismal level will only be visible after a series of molecular events. Morales et al. (2011) suggested that using subcellular biomarkers of stress is advantageous due

to their high sensitivity and fast response to stressors as compared to higher levels of organization (Morales et al. 2011). Sublethal cellular responses to stress can include rapid changes to metabolism, nutrient uptake, cell cycle growth, and the survival time of cells (Kroemer et al. 2010). Several methods have been suggested for quantifying sublethal stress, include the monitoring of reproductive habits and growth rates (Yousef & Courtney, 2003; Petes et al. 2008). Promise also resides in the form of stress protein quantification (Martin, 2000; Petes et al. 2008). Heat shock proteins (HSPs) are a class of molecular chaperones which aid in the protection/refolding of denaturing and aggregating proteins. HSPs are induced from a variety of stresses, including increased salinity, though they were initially discovered in the cells of organisms exposed to high temperatures. (Hochachka & Somero, 1984; Hill et al. 2012). During instances of biotic and abiotic cellular stress HSPs are rapidly upregulated, allowing them to serve as molecular indicators of stress (Lund et al. 2003; Lencioni et al. 2009; Hochachka & Somero, 1984; Zhao & Jones, 2012).

HSP70 has been demonstrated as inducible in the cells of insect larvae, making it a prime choice for our sublethal stress assessment protocol. De Jong et al. (2006) showed that chloride cells in the abdomen and gills of mayfly larvae expressed high levels of HSP70 in individuals impacted by road salt runoff. Stress protein expression across specific tissues and organs varies among species, individuals, and within different tissues of the body; also, the level of observed expression may be dependent on exposure time to a stressor or the time of year (Krebs & Feder, 1997; Singh & Lakhotia, 2000; Hyne & Maher, 2001). By establishing if/where HSP70 expression is concentrated, researchers may be able to interpret how osmotic stress is introduced and amplified in aquatic nymphs, such as by feeding, osmoregulation, or oxygen intake.

After choosing the mechanism by which stress would be assessed, we selected an organism capable of serving as a bioindicator. Bioindicators are organisms or environmental traits capable of serving as reliable indicators of environmental health (Stocker, 1980). Due to their importance in freshwater ecosystems, we chose to use macroinvertebrates. These animals are important to the structure and function of freshwater ecosystems, and their minimization or loss will potentially affect other trophic levels (Benbow & Merritt, 2004). Of the various macroinvertebrate groups, stoneflies (order: Plecoptera) are a favorable choice. This is because of their availability in streams during all seasons, the ease of collecting them, and their large size, which allows for examination of stress at the individual level (Gaufin & Tarzwell, 1952; Kohler et al. 1992).

Objectives

The goal of this project was to enhance biomonitoring efforts and early detection of thermal and salt stress on stream biodiversity in New Hampshire and to develop techniques that will provide an early-warning signal of ecosystems in jeopardy. Our project objectives were to:

1) **Evaluate differences in stream macroinvertebrate communities along a salt stress gradient.** This objective was met by evaluating macroinvertebrate community composition within ten 1st to 4th order wadeable streams across NH that varied along a salt gradient classified using snapshot water chemistry data from 2013, 2014, and 2016 as well as continuous monitoring of stream conductivity between 2013 and 2016.

2) **Evaluate differences in stream macroinvertebrate communities along a thermal gradient.** This objective was met by evaluating macroinvertebrate community composition within ten 1st to 4th order wadeable streams across NH that varied along a thermal gradient classified using 2016 snapshot and continuous monitoring of stream temperature.

3) **Evaluate sub-lethal osmotic stress in stonefly larvae** by quantifying heat shock protein (HSP70) expression in stoneflies (genus *Acuernaria*) using immunoblotting. This objective will be met by first conducting in-lab salt exposure trials using nymphal stoneflied to evaluate HSP expression in response to salt (NaCl) and thermal treatments.

4) **Compare and evaluate benthic macroinvertebrate sampling techniques and potential indicator taxa for salt stress.** The NH Department of Environmental Services (NHDES)– Biomonitoring program has adopted a rock basket approach for assessing water quality using indicator taxa and community metrics. We set out to compare the rock basket approach to kicknetting over the months of July –September/October to evaluate their ability to detect small changes in community composition that may be attributed to elevated salt or temperature.

This project had five main field and components: 1) field sampling of macroinvertebrates to provide community and population (family-level) metrics of ecological response, 2) laboratory based stress experiments to determine HSP induction thresholds for thermal-salt stress in two mayfly species, 3) field sampling and HSP expression assays of mayfly nymphs from streams that span a thermal and salt gradient to determine the utility of HSPs as biomarkers of stress in wild populations of mayflies, 4) continuous monitoring of conductivity, water level, temperature (stream and air), and 5) snapshot water chemistry (anion and cation) sampling to coincide with macroinvertebrate sampling.

Methods

Site selection
Field sites were selected in 2016 by using GIS to overlay the LoVoTECS network of stream monitoring sites with fish sample sites between 2009 and 2015. From this subset of NH streams, we selected sites based on median chloride concentrations derived from snapshot water chemistry data collected in May and July 2013 and July, Sept, Oct 2014. Our ten sites ranged

Sampling Locations

Sampling Sites
Chloride Level (mg/L)

- 2.88 - 8.07
- 16.87 - 26.94
- 57.93

PRW
DGB
MRL
CSR CBU HYB SNB
SUC
WHB
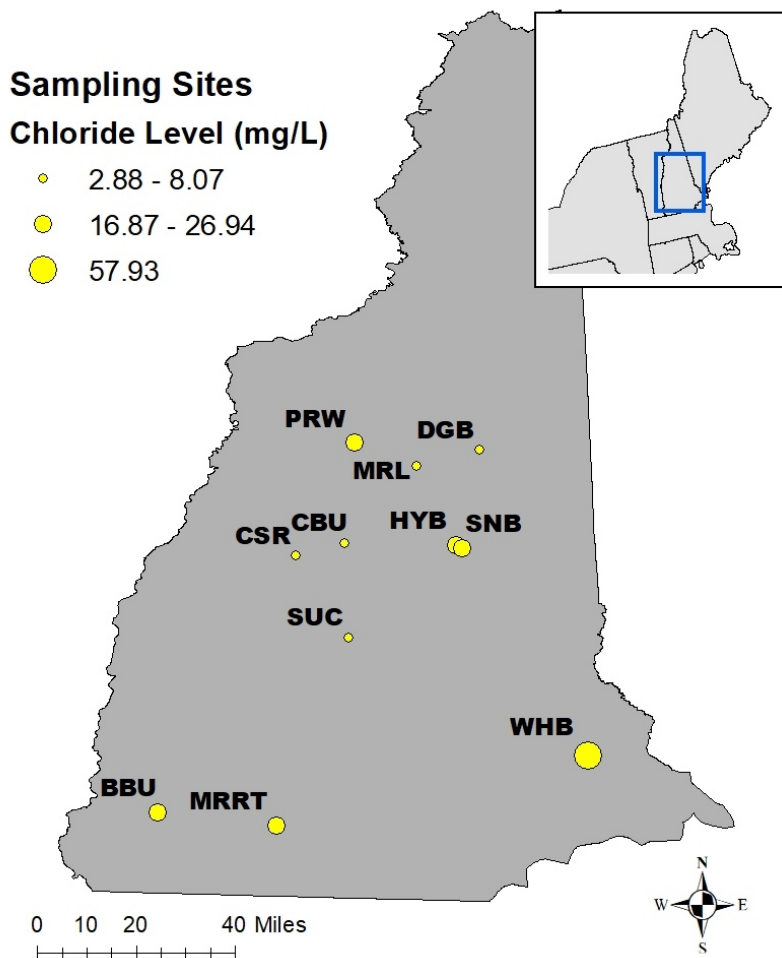BBU
MRRT

0 10 20   40 Miles

N
W E
S

Figure 1:Ten streams sampled for benthic macroinvertebrate and chloride between June and October 2017.

from 4.35-52.6 mg/L of Chloride during this period. The ten sites represent a range of human impact; some sites have roads and development, and some sites have little to no human impact. Two of our original sites, Mad River in Waterville Valley and Douglas Brook near the Kancamangus Highway, are located in the White Mountain National Forest. We noticed a strong correlation between chloride levels at these two sites as well as benthic macroinvertebrate communities. Therefore, we replaced the Douglas Brook site with a new stream reach on the Cockermouth River (Groton, NH) in 2017. The other stream sites are located near minor and major road systems, with minimal to moderate influence from road salts and other anthropogenic influences. The ten sites include: Halfway Brook and Shannon Brook in Moultonborough, Mad River in Waterville Valley, Beaver Brook in Keene, Wednesday Hill Brook in Lee, Pemigewasset River in Woodstock, Clay Brook in Plymouth, Cockermounth River in Groton, Otter Brook in Peterborough, and Sucker Brook in Franklin.

Our research team adopted NAWQA and EPA Rapid Biological Assessment macroinvertebrate sampling protocols for multi-habitat kicknet sampling. We sampled each study stream once every month beginning in late-May to September/October, 2017. At each site,

we selected a 100-meter reach that was largely representative of the stream habitat. This 100-m reach was established in close proximity to continuously logging specific conductance, water temperature, and water level sensors; most sites consisted of sample reaches that were 50-meters upstream and 50-meters downstream, or, where that was not feasible, 25-meters and 75-meters. We sampled 10 kicks using a 500 μm net over the 100-meter stream reach, sampling different habitats in approximate proportion to their representation of the total surface area of the reach. We determined this by assigning a percentage of each habitat type (cobble, sand, or large woody debris) totaling 100%. In cobble substrate/habitat, we chose to kick in riffles or runs. In sand substrate and habitat, we mainly kicked in runs and slow moving water since that is the main stream morphology for this type of habitat. We placed all macroinvertebrates in labeled containers with 70% ethanol for preservation. If there were any predator macroinvertebrates, such as the family Corydalidae, then we used an additional container to store the predators.

In addition to kicknetting, we adopted the NHDES biomonitoring program rock basket approach for macroinvertebrate sampling. At each site, we deployed 3 rock baskets side-by-side in a cobble and riffle habitat in close proximity to the continuously logging sensors during the July sampling period. We collected rock baskets roughly after eight weeks in mid to late September to later compare results with NH DES Biomonitoring Program's annual assessments. Our rock basket collection was similar to the NHDES sampling protocol, which included four, 5-gallon buckets, 3 of which will hold the rock baskets themselves, and one bucket to rinse and store the rocks that have been examined. We filled three buckets with stream water a quarter full and facing upstream with the opening facing towards the rock basket. One person lifted each basket into the bucket, making sure to catch any debris that comes loose from the basket. The research team thoroughly examined every rock in each basket, and the water in the bucket was filtered through a 500 μm sieve. We placed all macroinvertebrates in rock basket labeled containers separately to the kick net samples, and stored in 70% ethanol to be preserved. We labeled containers with the correct site name and date sampled. Samples were transported back to Plymouth State University for identification and enumeration.

Macroinvertebrate Identification

Preserved field samples were identified to family using NAWQA and EPA protocol for macroinvertebrate sorting and identification. We used Voshell (2002) *A Guide to Common Freshwater Invertebrates of North America,* Merritt et al. (2008) *An Introduction to the Aquatic Insects of North America 4th Edition, and* Peckarsky et al. (1990) *Freshwater Macroinvertebrates of Northeastern North America* to aid in the identification of benthic macroinvertebrate families.

Community data analysis

We assessed community composition using two approaches, *a)* the traditional biomonitoring rapid assessment approach focusing on composition at the order level (e.g. percent EPT) and *b)* relative abundance of one tolerant and intolerant three families based on NHDES and Carlisle et al. (2008) tolerance values. We graphed site-level family richness and relative abundance for each site over the three sampling periods. We then evaluated the relationship between order and family-level metrics and a suite of potential explanatory variables using multiple linear regression. The lack of a clear and consistent relationship between chloride and the community metrics in 2016 prompted us to take a multiple linear regression approach to better understand the influencers of the observed macroinvertebrate communities (Mazzone

2018). We took a stepwise parameter selection approach that included the following explanatory variables: discharge, stream area, as well as snapshot measures of water temperature (snapshot), pH, dissolved oxygen, chloride, and sodium. Sample month was also included because there is uncertainty in the timing of emergence for all families observed. We included data from the 2016 season to provide a multi-year assessment. The metrics and explanatory variables found in the best fit models of each are summarized in Table 1. In addition, we graphed a comparison of cumulative measures of family level richness (within and across orders) and relative abundance of indicator families observed in rock baskets retrieved during September/October sampling and kicknet samples from the same sampling period.

Table 1:

				Explanatory Variables								
	Response Variables	Site	Month	Year	Chloride	DO	pH	Reach Area	Latitude	Water Temp	Elevation	Fluoride
Common Biomonitoring Metrics	Total Family Richness	x	-	+	+	-	+					
	EPT Taxa Richness		-	+				+	+	+	+	
	Ephemeroptera Family Richness	x	-							-		
	Plecoptera Family Richness		-	+						+		
	Tricoptera Family Richness					-	+					+
	% EPT			+				+				+
	% Ephemeroptera		-					+		-	+	-
	% Plecoptera	x	-					-				
	% Tricoptera		+	-	+			-	+	+		
	% Chironomidae								-			
	% Diptera	x			-					-		
Family Level Responses	Chloroperlidae Relative Abundance[IT]	x	+									
	Leuctridae Relative Abundance[IT]		-		+							
	Philopotamidae Relative Abundance[IT]	x										+
	Simuliidae Relative Abundance[T]	x	-	+								
	Rhyacophiidae Relative Abundance[IT]	x										
	# Positive		2	5	3	0	2	3	3	2	2	3
	# Negative		8	1	1	2	0	2	1	3	0	1
	Total	8	10	6	4	2	2	5	4	5	2	4

HSP70 expression:

    To test the utility of HSP70 in *Acroneuria* (Plecoptera, Perlidae) nymphs as a sub-lethal indicator of salt stress, our research sought to determine whether different combinations of NaCl dosage, temperature and time would cause differences in HSP70 expression. To answer this question, we conducted exposure experiments, exposing Perlidae nymphs to various levels of

NaCl and higher temperatures, then quantified the HSP70 response using Western blotting. Western blotting allows for the quantification of gene expression by measuring protein abundance from a given tissue. We predicted that HSP70 expression would show a marked increase with higher dosages of NaCl, independent of trial temperature, with higher levels attained over longer exposure times. Additionally, we predicted that interactions of higher temperature and NaCl dosages would result in the greatest expression of HSP70. The result of this approach was a measure of HSP70 related to experimental treatments and exposure time that could be assessed statistically.

Our local stream site for Perlidae collection was Clay Brook, Plymouth, NH (see Figure 9). This site was chosen for the high abundance of perlid stoneflies observed in previous seasons. Being a short drive from the laboratory, it also provided short transport times for live nymphs, reducing the chance of HSP70 induction through handling. Sampling of stream ion concentrations showed a low level of Cl^- (4.03 and 4.83mg/L, July and September 2017), suggesting little present influence of NaCl.

Protocol Development

Collect Nymphs

| HSP70 Expression across body regions | HSP70 Expression with NaCl &Temp. Exposure | HSP70 Expression in Field Specimens |

Dissect into sections ← Exposure trials (72 hrs) → Sacrifice in field

Protein extraction
BCA quantification
Western blotting
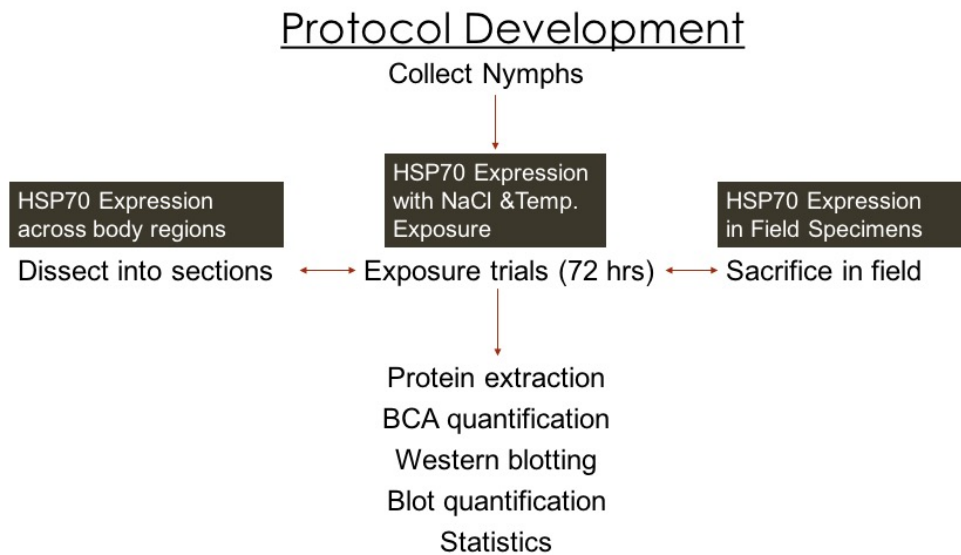Blot quantification
Statistics

Figure 2: Overview of HSP70 analysis

We collected stonefly nymphs from May through July 2017 from Clay Brook using kicknetting, with stream temperature recorded during each visit. Nymphs were transported to the laboratory in a portable cooler to avoid heat stress. Stream water and leaves were collected from the site for micro aquaria to maintain consistent pre-treatment ionic conditions and provide a food source. Specimens and water were transferred to micro aquaria setups consisting of one-liter beakers with battery powered-bubblers as tanks for collected stoneflies (Kennedy et al. 2004; Echols et al. 2013). Screen netting was fitted into each tank to provide an attachment substrate. We exposed stonefly nymphs to various levels of NaCl and temperature (see Table 2). We acclimated nymphs for 72 hours at 4 and 21°C prior to trials to rule out HSP70 expression due to handling/travel. Total protein was extracted from five nymphs to provide a measure of baseline HSP70 expression. Nymphs were exposed in a series of trials to 0 mg/L, 2500 mg/L, and 4000 mg/L NaCl dissolved in 50mL of diH_2O. Specimens were also exposed to temperatures of 4, 21, and 28°C. These temperatures and NaCl dosages were chosen to elicit a stress response

without lethal harm to the experimental population. Following each application, nymphs were sacrificed at the 1, 24, 48, and 72 hour marks. Head samples were used for all blotting and analysis in this experiment following observed HSP70 concentration within this body region. Refer to Chapter 2 in Fruit (2018) for a detailed discussion of this preliminary work.

Figure 3. Location of Clay Brook, sample site for Perlidae in Plymouth, NH

Quantification of HSP70

  Western blotting is a research technique for identifying target proteins from a mixture. Electrophoresis through a gel medium is used to separate extracts based on molecular weight/size. Proteins are then transferred to a membrane, resulting in bands of protein which can be identified by incubation with a primary and secondary antibody and subsequent substrate development (Mahmood & Yang, 2012). Blots were visualized using a BioRad ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Inc.), at which point images were exported as high resolution images. For more specific protocol, please refer to Fruit (2018).
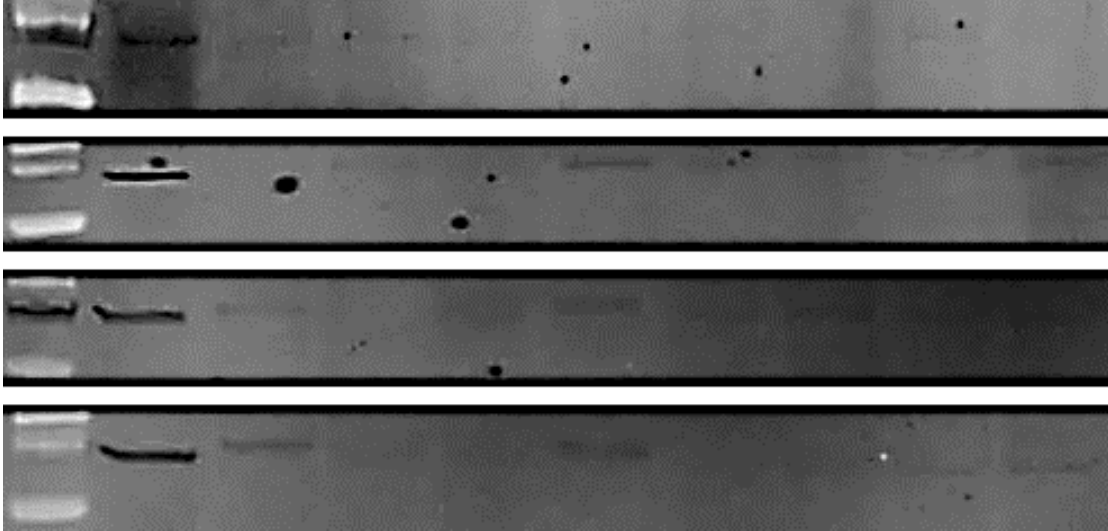
Figure 4. Representative western blots of head samples extracted 1, 24, 48 and 72 hours after treatment conditions commenced (ascending order from top).

We used ImageJ software to quantify pixel counts of HSP70 bands. Our method was similar to that of Taylor & Posch (2014), using a control sample loaded onto every blot to standardize between all samples. Background noise from the target protein of each sample was subtracted in ImageJ, and the resulting output was multiplied by a ratio of the loading control for each sample and the inter-blot control. This normalized value is referred to as the normalized density to the loading control (NDL). The NDL of each experimental sample was then divided by the NDL of each inter-blot control, yielding a fold difference (FD) value from the amount of control expression (Taylor et al. 2013; Taylor & Posch, 2014). We used a sample of *Drosophila* protein in this study as the inter-blot control and our positive control. For the loading control of each experimental and the inter-blot sample, we used total protein of each lane stained by Ponceau-S. Hereafter, FD values of experimental samples will be referred to as HSP70 expression.

Trial Time (hrs)	N	NaCl (mg/L)	Temperature (℃)
0	5	0	4
1	2	2500	4
	4	4000	4
	1	0	21
	2	2500	21
	2	4000	21
	3	0	28
	2	2500	28
24	2	4000	4
	2	0	4
	1	2500	21
	2	4000	21
	2	0	21
	1	2500	28
	2	4000	28
48	1	0	4
	2	2500	4
	2	4000	21
	2	0	21
	2	2500	21
	2	4000	28
	1	0	28
72	2	2500	4
	3	4000	4
	2	0	21
	2	2500	21
	2	4000	21
	3	0	28
	2	2500	28
TOTAL	61		

Table 2 - Total Salt Trial Subjects. Including trial time, temperature and NaCl dosage and number of individuals per treatment.

Data analysis

We had 61 samples in total, for multiple combinations of 5 different trial times, 3 different NaCl dosages and 3 different trial temperatures. Observed HSP70 expression ranged from FD values of -0.0592 (compared to observed control) to 0.589, nearly a ten-fold difference across treatments (see Appendix C). We graphed box-plots to illustrate differences in HSP70 across trials representing unique combinations of NaCl dose, temperature and exposure time. To best visualize all treatment groups, trial temperature and NaCl treatments were concatenated and used as one factor alongside trial time (see Figure 11). We created ANOVA interaction plots visualizing model effects and mean distributions of HSP70 expression indicating interactions of combinations of explanatory variables. We then graphed boxplots of HSP70 expression in relation to time and either NaCl or temperature to visualize which factors, if any, corresponded (Figure 5). To verify observed effects, we ran non-parametric ANOVA (Kruskall-Wallis) tests to test for statistical differences in observed HSP70 expression explained by NaCl dose, temperature and exposure time in isolation. Each experimental variable was run independently,

with other variables collapsed. To address the possibility of factors interacting to influence variation of HSP70 expression, two-way ANOVA was used to examine variation with respect to combinations of NaCl dosage, trial temperature and exposure time. Finally, multiple-linear regression was used to corroborate ANOVA results for the observed relationship of these factors on variation in HSP70 expression.

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<u>Principal Findings & Significance</u>

<u>Chloride concentrations</u>
       Monthly snapshot water chemistry samples confirmed the initial classification of streams (based on snapshot sampling in 2013 and 2014) and were similar to those reported from 2016, with the exception of consistently lower concentrations at the Beaver Brook site (BBU) in Keene, NH. The new site for 2017 turned out to be another low concentration site, despite extremely close proximity to road. As also seen in 2016, Wednesday Hill Brook (WHB) had the highest Cl concentrations across our study sites and over time. Even this, our highest observed chloride concentration, was substantially below the EPA's chronic toxicity concentration of 230 mg/L. Chloride concentrations increased at most sites between July and September/October, which we believe is attributed to lower water levels of which groundwater likely comprises a larger portion of stream water. These findings support the findings of Daily et al. (2009).
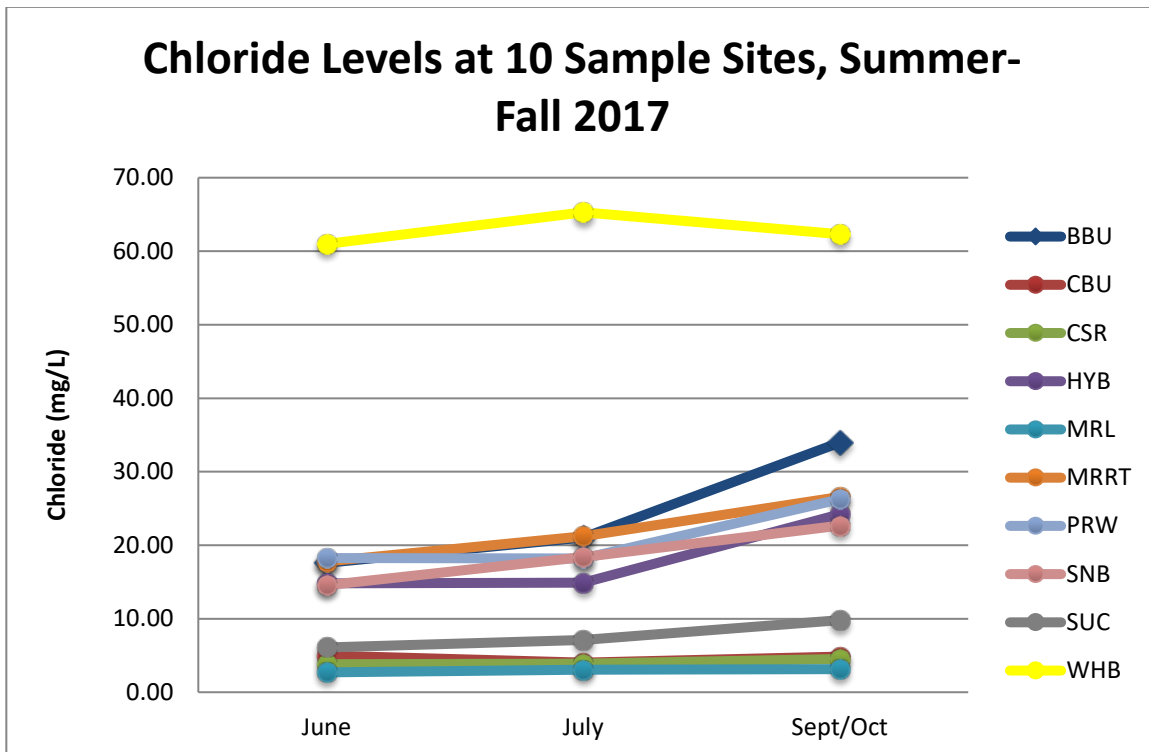
Figure 5: Snapshot sampling of chloride in 10 sample streams between June and September/October 2017.

Community Composition

We found that chloride rarely explained a significant portion of the observed variation in the aforementioned community metrics in summers 2016 & 2017 (Table 1). Of the four metrics that were significantly related to chloride, only % Diptera was negatively related to chloride, which was opposite the expectation. Among the unexpected, yet significant relationships with chloride were positive observed relationships with the percent Plecoptera (Figure 6). Further analysis indicated that this pattern, also observed in 2017, was largely driven by the relative abundance of Leuctridae, a plecopteran family previously categorized as intolerant to poor water quality (Figure 7). Interestingly, few other Plecoptera families were present when chloride concentrations exceeded 30 mg/L and this was evident with the negative trend observed between chloride and Plecoptera richness (Figure 8). We had adopted Leutridae as an intolerant indicator family based on published tolerance scores. Our observations don't support the perceived sensitivity of this family. This may be driven by unknowingly sampling a single genus or species that is more tolerant than others in the family. Alternatively, it could suggest that Leutrids are less sensitive to ionic concentrations than other sources of water quality stress, which would imply that they are a poor bioindicator choice for monitoring the effects of salinization.

We observed was significant inter-annual and intra-annual variability with generally higher metric values in 2017 and lower values as the season progressed from June to Sept/October. Ephemeroptera richness, percent Ephemeroptera, and percent Diptera were negatively related to observed water temperatures, whereas percent Tricoptera and EPT Richness were positively related to observed water temperatures. This suggests that Ephemeroptera composition may serve as a strong bioindicator of water temperature stress, even at the relatively low temperatures observed during our study. We did not find significant relationships between chloride or temperature and the majority of our pre-identified indicator families, with the exception of Leuctridae.

Overall, our observed communities seem to be shaped more by natural site-level variability in elevation, latitude, reach area, and time than by ionic concentrations or temperature, suggesting that chloride concentrations are not negatively affecting the streams in this study.
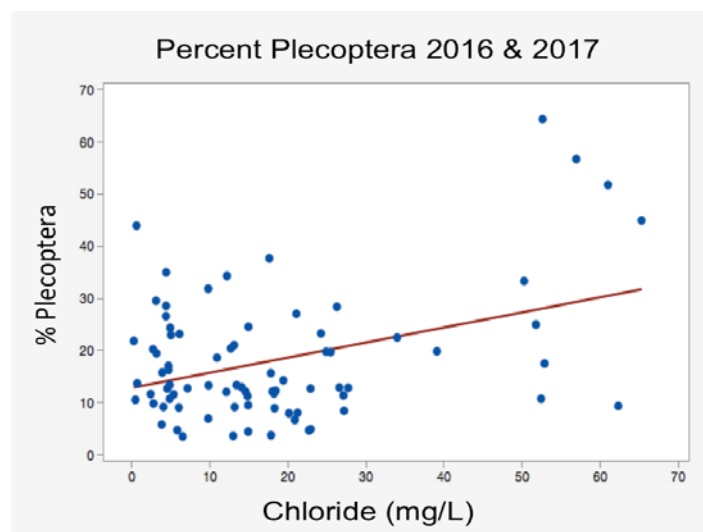

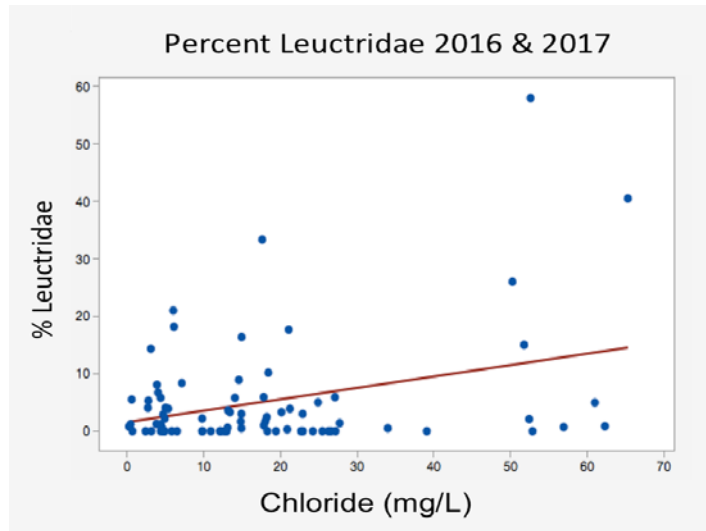*Figure 6: Relative abundance (%) of Plecoptera individuals*

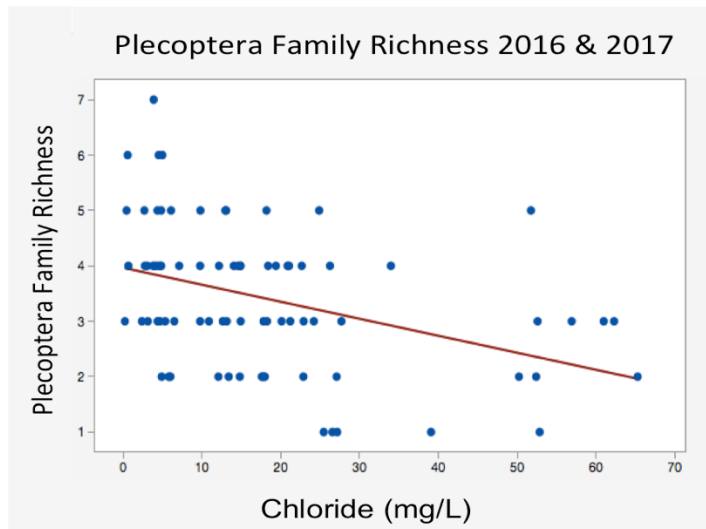*Figure 7: Relative abundance (%) of Leuctridae individuals (order: Plecoptera)*

Figure 8: Plecoptera richess (i.e.number of Plecoptera families)

Rock baskets vs. Kicknet sampling

We compared communities observed using kick netting and rock basket methods to assess whether important metrics were equally represented. Our results suggest that kick netting with 10 sets over 100m yielded higher total abundance of macroinvertebrates and higher richness of all families, EPT families collectively and by order at most sites (Figures 9-14). The difference in richness measures is clear evidence that the rock baskets do not reflect the full diversity within a defined reach. While this is not completely necessary, depending on the

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objective of the assessment, it could be problematic if the method is biased away from potential indicators of chloride stress. For example, individuals in the Philopotamidae and Simuliidae families were rarely found in a rock basket sample (Figures 13 & 14). The lack of taxanomic representation in rock baskets will also influence the relative abundance of those groups present. For example, percent Chironomidae (was consistently much higher in rock basket than kick net samples at our sites (Figure 15). We also noticed that difference between the rock basket and kick net samples was among the greatest at Wednesday Hill Brook (WHB), the site with consistently higher chloride concentrations. In pursuit of a reliable bioindicator taxa, we must also consider the best method for sampling.
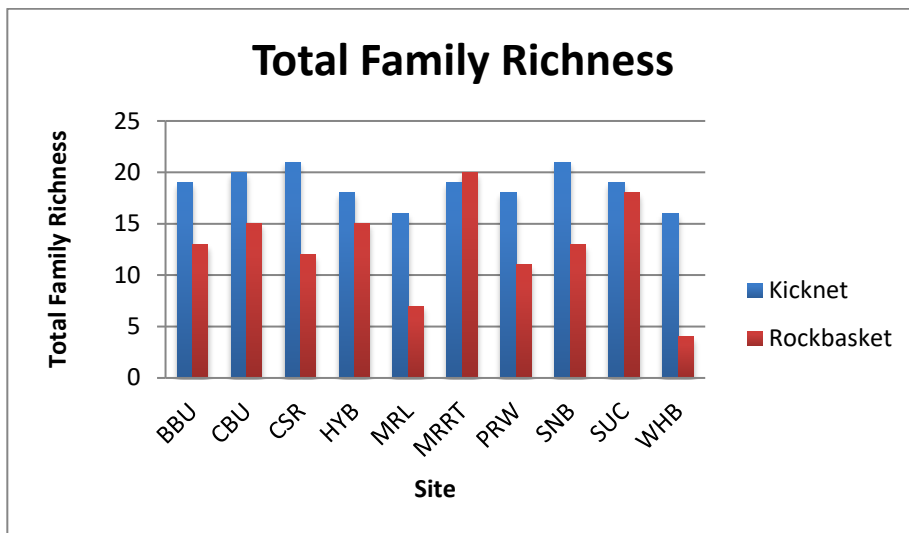
Figure 9: Total family richness measured using kicknet and rock basket sampling methods.
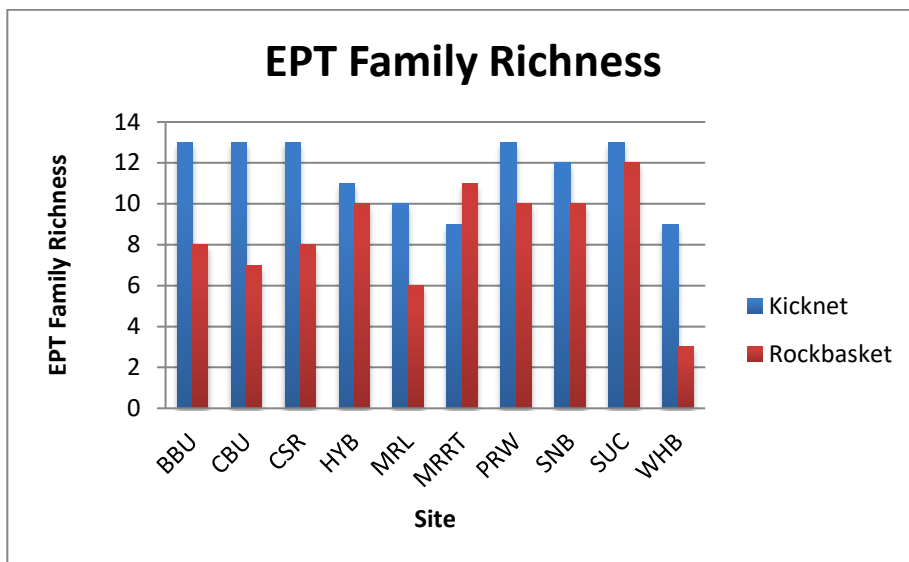
Figure 10: EPT family richness measured using kick net and rock basket sampling methods.
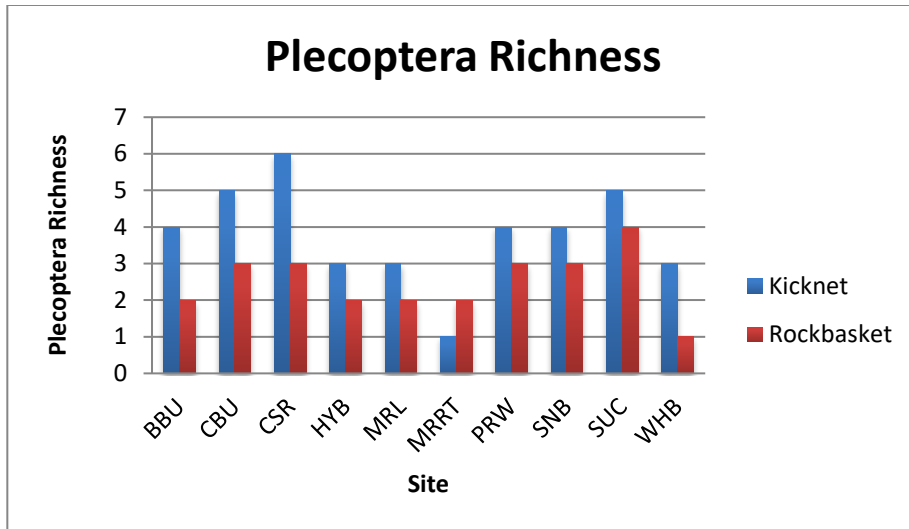
Figure 11: Plecoptera richness measured using kick net and rock basket sampling methods.
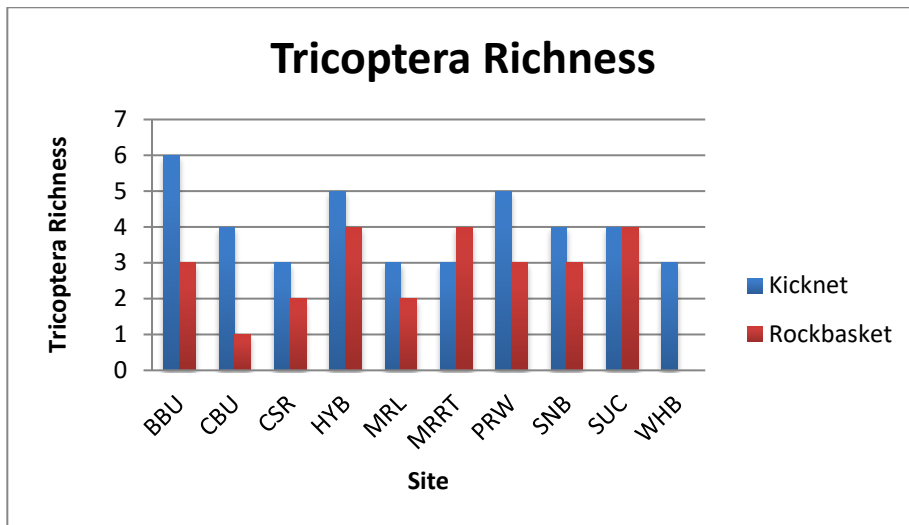

Figure 12: Tricoptera richness measured using kick net and rock basket sampling methods.
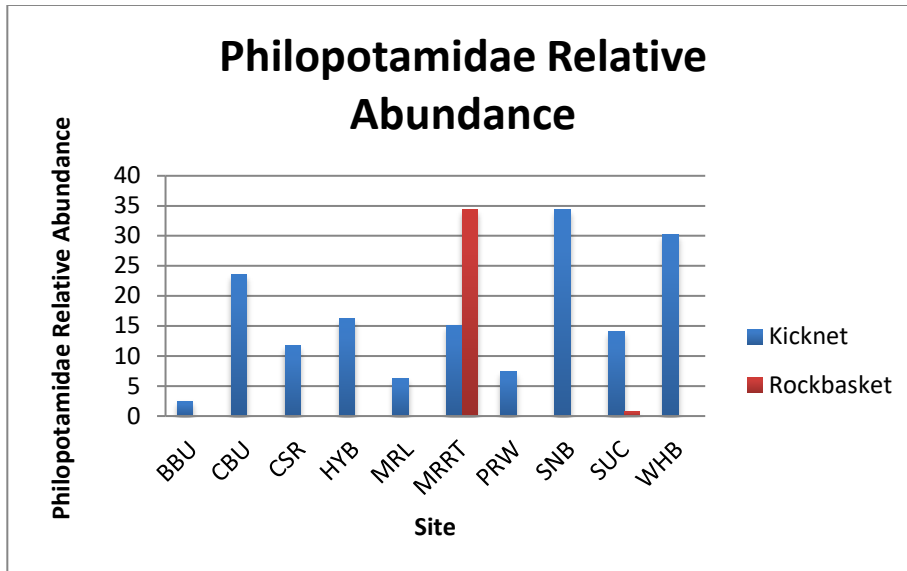
Figure 13: Relative abundance of Philopotamidae measured using kick net and rock basket sampling methods.
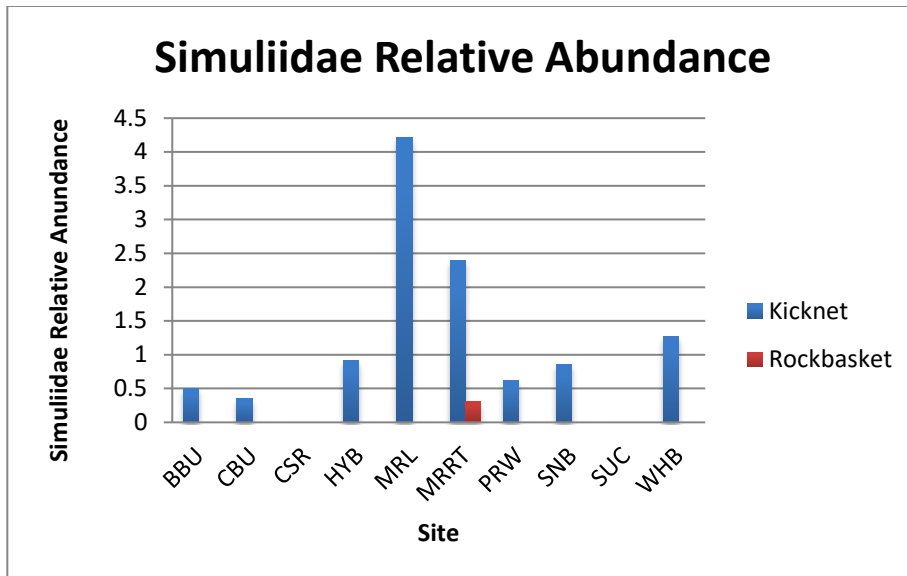

Figure 14: Relative abundance of Simuliidae measured using kick net and rock basket sampling methods.
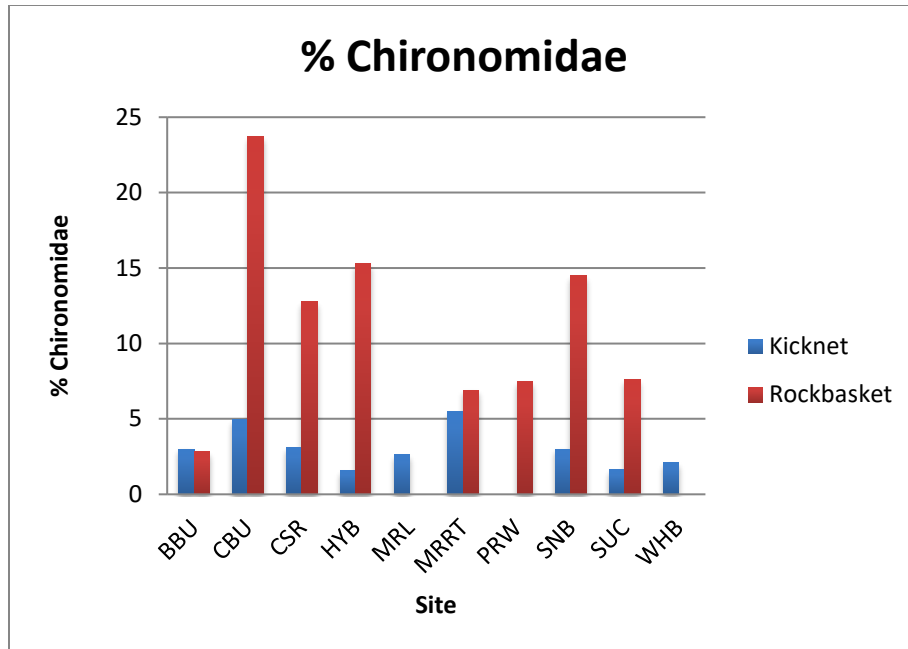
Figure 15: Relative abundance (%) of Chironomidae measured using kick net and rock basket sampling methods.

HSP Analysis

Alone, NaCl and temperature variables did not explain the observed variation in HSP70 expression (N = 61, p-value = 0.9548 and N = 61, p-value = 0.3508); however, there was an observed and disproportionate uptick in HSP expression at for samples held at 21 and 28° C (Figure 16).
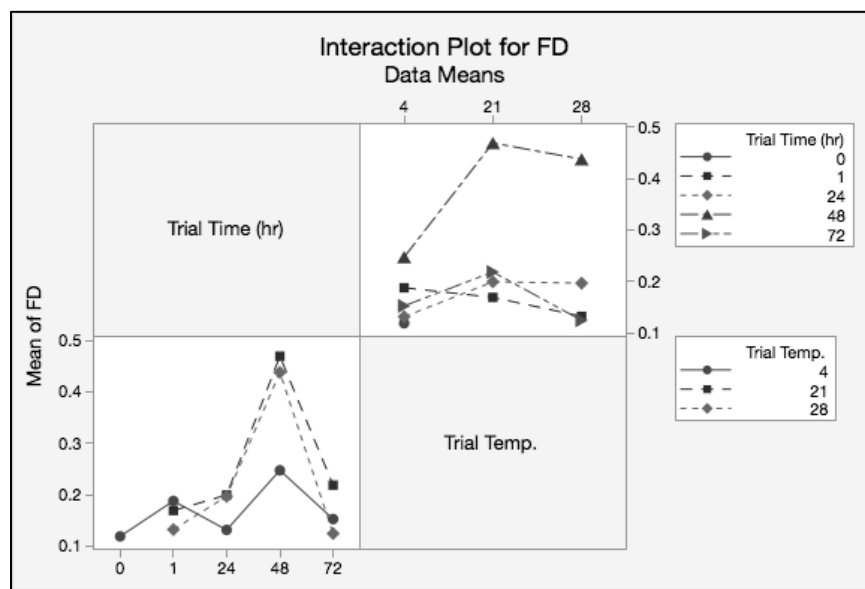

Figure 16: Interaction plot illustrating the observed relationships between exposure time and temperature.

Exposure time was a significant treatment factor (N = 61, p-value = 0.0194). This was supported by observed levels of HSP70 expression after 48 hours (Figure 16 and 17). Likewise, the two-way ANOVA analysis also suggested exposure time partially explained the increase in HSP70 expression (N = 59, p-value $\cong$ 0.0044). The results from our various ANOVA analysis were corroborated by running a multiple linear regression of NaCl dosage, trial temperature and exposure time as a categorical variable in which 48 hours of exposure time was found to have significantly higher HSP70 expression (N = 60, p-value $\cong$ 0.005).
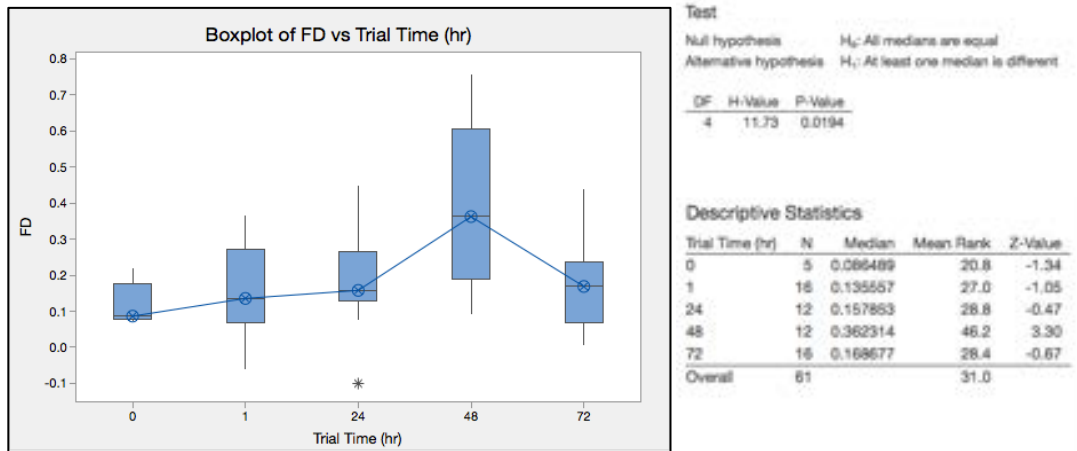

Figure 17 - Kruskal-Wallis ANOVA results showing significant differences between 48hr extractions and other time periods.

Neither NaCl or temperature explained the variation observed in HSP70 expression at the treatment levels tested; however, our results do indicate that exposure time explained a significant portion of observed variability in the HSP70 expression in stonefly nymphs. Expression noticeably increased in specimens exposed to 21 or 28°C, peaking between 24 to 48 hours or 48-72 hours and returning to baseline levels by 72 hours (backed by Kruskal-Wallis ANOVA, Two-Way ANOVA and Multiple-Linear Regression).

Response to NaCl

The lack of a statistically significant treatment effect could be explained by several factors, one of which is the particular chemical composition to which specimens were exposed. Molecular grade NaCl was used as a proxy for road salt; however, this lacks many of the additives used for road deicing (such as abrasives) which may have synergistic effects harmful to aquatic macroinvertebrates. Additionally, NaCl (or free Cl- ions, for that matter) may not be as acutely toxic to aquatic macroinvertebrates as previously thought. Instead, the toxicity may be attributed to other free ions released from soil sediments by the constituents of road salts. NaCl has been shown to mobilize metals within soils, both through complexing with Cl⁻ ions and cation exchange with Na⁺ ions (Benjamin, 2002; Norrstrom and Jacks, 1998; Backstrom et al. 2004). A third potential explanation might be that aeration from the bubbler in the micro aquaria reduced exposure to toxicants as compared to stagnant/stiller water (Sanders & Cope, 1968.) This was unavoidable in our experiment, as we sought to recreate stream conditions as closely as possible. A final potential explanation was the small sample size of each specific combination of trial factors preventing a proper analysis of NaCl-related stress. This was due to the various

temperatures and exposure times, meaning only one to four specimens per NaCl dosage; ideally, each NaCl dosage would have at least 20 representative specimens.

Response to temperature

The temperature component of these trials is also interesting and unexpected. Our results indicate that specimens exposed to 21°C reached the highest levels of HSP70 expression at 48 hours, while those exposed to temperatures of 28°C achieved lower levels of HSP70 expression at 48 hours and fell to lower levels than both 4 & 21°C specimens after 72 hours. This was based entirely off data visualization and means observed in interaction plots, as statistical tests discounted temperature as significantly influencing variation in HSP70. At times, organisms exposed to the high levels of harmful contaminants in an experiment have lower levels of HSP70 expression than those exposed to lower levels (Pyza et al. 1997 & Kohler et al. 1992). Similarly, Pyza et al. (1997) found the greatest mean HSP70 levels in heat-treated centipedes to be at 15°C, and not at 5 or 25°C. The HSP70 response to temperature is well-known, but still subject to variability. Threshold temperatures for the activation of HSP genes is known to vary over the lifetime of an individual and is subject to thermal acclimation to an environment (Buckley et al. 2001). Brook Trout, for example, express high levels of HSP70 at the same average temperature across two different years of study (Chadwick Jr. et al. 2015). This could suggest that past exposure to temperature stress had hardened nymphs against physiological stress from heat. More specifically, it may be that expression of HSP70 in stonefly nymphs under these experimental conditions was exhausted by temperatures of 28°C after an attempted spike to achieve homeostasis by 48 hours, and subsequently crashed by 72 hours. The observation that those specimens' HSP70 expression exposed to 21°C rose higher at 48 hours and finished higher at 72 hours could suggest that this temperature exposure was not severe enough to exhaust the HSP70 response.

Alternative Influences

The importance of the exposure history of individuals should not be overlooked in a study using HSPs. While we attempted to minimize any stressors influencing the expression of HSP70, it is difficult to completely account for past influences. Hochachka & Somero (1984) point out that there is significant adaptive variation of the heat shock response from recent thermal history and selective forces. Moreover, the threshold of HSP induction varies due to thermal acclimation, and can vary over the lifetime of a single individual. HSP70 can build up within cells following repeated gradual warming events (Buckley et al. 2001).

A related problem lies with the possibility of individual variation of the heat shock response among organisms collected in the same environment. This is because past exposures to stressful conditions are capable of acting as evolutionary forces upon populations (Sørensen et al. 2003). Feder & Hofmann (1999) point out that while variation in HSPs can be due to seasonal variation or acclimation to stressors, natural variation will also be present from genetic differences of individuals. In addition, variation in the tolerance to ionic changes may manifest at the species level, rather than the genus level at which we worked.

Conclusion

Evidence from our two-year study suggests that streams in central New Hampshire remain relatively unstressed by salt additions attributed to road salt. All streams monitored were under the EPA's chronic toxicity concentration. However, our study has found that not all

Plecoptera taxa are "sensitive" or intolerant to salinity stress. We found a positive relationship between Leuctridae (family in order of Plecoptera) and chloride concentrations during both study seasons, despite a decrease in Plecoptera family richness. Further, the molecular level analyses found that *Acroneuria* (another genus in the family of Perlidae in order of Plecoptera) had an exceptionally high tolerance to NaCl in lab settings (4000 mg/L). This leads us to believe that sensitivity to salt stress likely varies at the family, if not genus/species, level. If so, traditional biomonitoring metrics that focus on the relative abundance of EPT taxa may not be fine enough resolution to detect stress. Finally, our comparison of benthic macroinvertebrates detected in rock baskets to that of kicknets suggest that rock baskets do not fully reflect the biota present and that they may select against families/genera with salt sensitivity by nature of the method alone. Further research is needed to compare rock basket taxa to kicknet and to understand which families are more vulnerable to salt stress in New Hampshire. To accomplish such, we suggest more studies in areas where chloride concentrations are higher – mainly in southern NH; however, it is also important to continue to monitor streams in central and northern NH to maintain healthy systems.

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Notable Awards:
Dr. Amy Villamagna was honored with the Helen Abbott Endowed Professors of Environmental Studies (2016-2020) for her research on the environment and engagement of students in research.
Katerina Crowley was awarded the Marapesse Scholarship in 2017 for her participation in this research on Sucker Brook, a tributary to Webster Lake (NH).
Katerina Crowley was awarded second place in student poster competition at the 2018 New England Association of Environmental Biologists in Devens, MA.

Publications and Presentations:
2018
Fruit, R. K. 2018. 'A Novel Approach to Biomonitoring: Identifying Communities At-risk from Salinization Using HSP70 Expression in Stonefly Nymphs'. Master of Science in Environmental Science & Policy. Plymouth State University. Plymouth, NH (USA)
Mazzone, M. 2018. 'The Impacts of Chloride on Macroinvertebrate Communities in New Hampshire Streams'. Master of Science in Environmental Science & Policy. Plymouth State University. Plymouth, NH (USA)
Fruit, R., A. Villamagna, B. O'Donnell. 2017. HSP70 Induction Following Salt and Temperature Stress in the Stonefly *Acroneuria* – A Novel Approach to Biomonitoring. New England Association of Environmental Biologists annual meeting (Devens, MA). Oral presentation
Kat Crowley. Assessing ecological stress from chloride in New Hampshire streams at community and population levels. New England Association of Environmental Biologists annual meeting (Devens, MA). Poster presentation
Fruit, R., A. Villamagna, B. O'Donnell. 2017. Identifying Communities at-risk from Salinization Using HSP70 Expression in Stonefly Nymphs. New Hampshire Water and Watersheds conference. Poster presentation
Kat Crowley. Assessing ecological stress from chloride in New Hampshire streams at community and population levels. New Hampshire Water and Watersheds conference. Poster presentation
2017 (2016-17 funding cycle)
Duquette, R., A. Villamagna, B. O'Donnell. 2017. *Assessment of Mayfly, Stonefly and Caddisfly abundances in relation to chloride in New Hampshire streams.* New England Association of Environmental Biologists annual meeting (Hartford, CT) *[poster printed but not presented due to poor blizzard travel conditions]*
Lafortune, T., A. Villamagna, B. O'Donnell. 2016. *Air and Stream Temperature Relationships and Influence on Macroinvertebrate Communities in New Hampshire.* New England Association of Environmental Biologists annual meeting (Hartford, CT) *[poster printed but not presented due to poor blizzard travel conditions]*
Fruit, R. , A. Villamagna, B. O'Donnell. 2017. *Quantification of HSP70 Expression in Mayflies: A Novel Bioindicator of Road Salt Pollution.* New England Association of Environmental Biologists annual meeting (Hartford, CT) oral presentation

Mazzone, M. A. Villamagna, B. O'Donnell. 2017. *Assessing Salt Stress In Selected NH Streams at the Community Level For Macroinvertebrates.* New England Association of Environmental Biologists annual meeting (Hartford, CT) *[oral presentation prepared but not presented due to poor blizzard travel conditions]*

Duquette, R., A. Villamagna, B. O'Donnell. 2017. *Assessment of Mayfly, Stonefly and Caddisfly abundances in relation to chloride in New Hampshire streams.* Plymouth State University Showcase of Excellence (poster)

Lafortune, T., A. Villamagna, B. O'Donnell. 2016. *Air and Stream Temperature Relationships and Influence on Macroinvertebrate Communities in New Hampshire.* Plymouth State University Showcase of Excellence (poster)

Fruit, R., A. Villamagna, B. O'Donnell. 2017. *Quantification of HSP70 Expression in Mayflies: A Novel Bioindicator of Road Salt Pollution.* Plymouth State University Showcase of Excellence (oral presentation)

Mazzone, M., A. Villamagna, B. O'Donnell. 2017. Assessing Salt Stress on Macroinvertebrate Communities in NH Streams. Plymouth State University Showcase of Excellence (oral presentation)

2016 (2016-17 funding cycle)

Fruit, R. , A. Villamagna, B. O'Donnell. 2016. *Stress Protein Expression: An Early Warning Sign of Freshwater Community Degradation via Road Salt Runoff in New Hampshire* (poster), 2016 NH Water and Watersheds Conference in Plymouth, New Hampshire

Duquette, R., A. Villamagna, B. O'Donnell. 2016. *Assessment of Mayfly, Stonefly and Caddisfly abundances in relation to chloride in New Hampshire streams.* Hubbard Brook Research Experience for Undergraduates Symposium (Thorton, NH)

Lafortune, T., A. Villamagna, B. O'Donnell. 2016. *Air and Stream Temperature Relationships and Influence on Macroinvertebrate Communities in New Hampshire.* Hubbard Brook Research Experience for Undergraduates Symposium (Thorton, NH)

Duquette, R., A. Villamagna, B. O'Donnell. 2016. *Assessment of Mayfly, Stonefly and Caddisfly abundances in relation to chloride in New Hampshire streams.* EPSCoR Research for Undergraduates Symposium at University of New Hampshire (Durham, NH)

Lafortune, T., A. Villamagna, B. O'Donnell. 2016. *Air and Stream Temperature Relationships and Influence on Macroinvertebrate Communities in New Hampshire.* EPSCoR Research for Undergraduates Symposium at University of New Hampshire (Durham, NH)

Number of students supported*:* 2 MS students, K. Crowley and M. Hirschler. 4 undergraduate students, S. Bevier, T. Lafortune, M. Conlon, J. Burdick were affiliated with the project through university match and research collaboration. Not all received direct funding from NH WRRC.

Number of faculty supported: Assistant professor, Amy Villamagna (Ph.D.) and Associate professor, Brigid O'Donnell (Ph.D.) were affiliated with the project through university match and research collaboration.
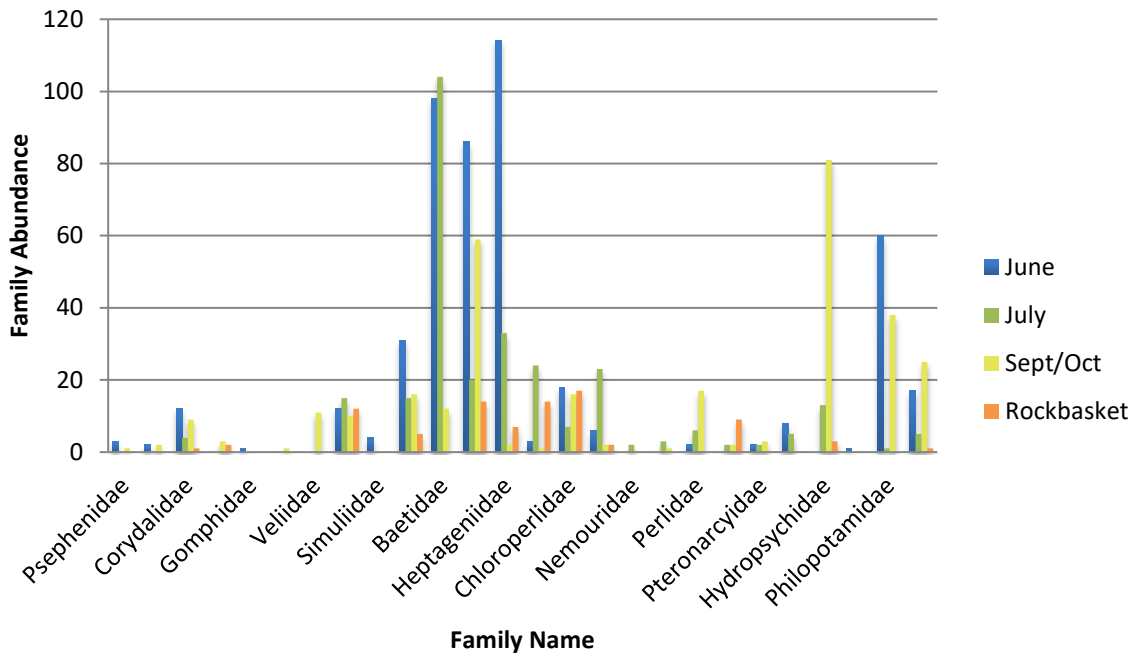
APPENDICES

Appendix A: Within season comparison of family level abundance observed through kicknet sampling for benthic macroinvertebrates at all ten study sites.
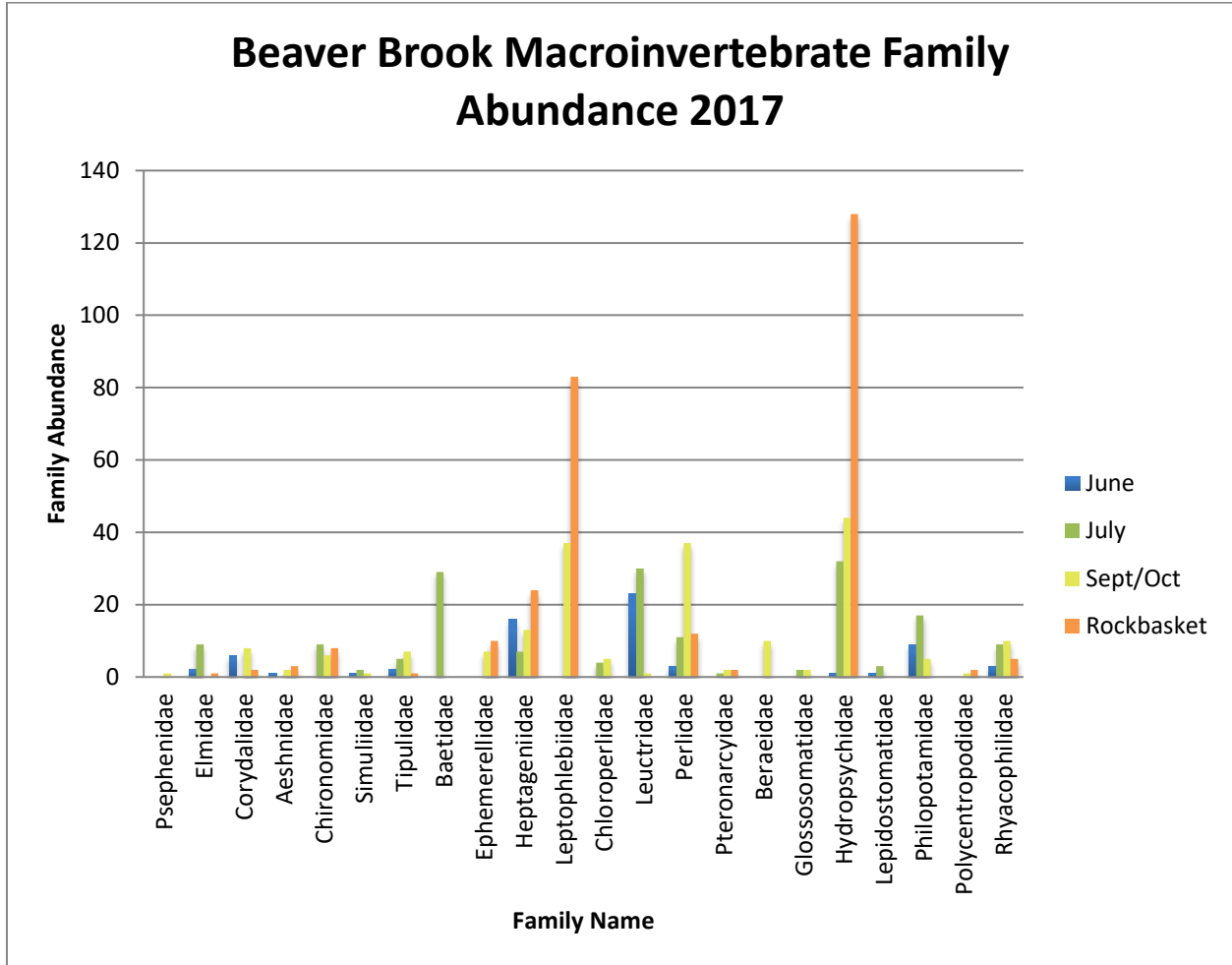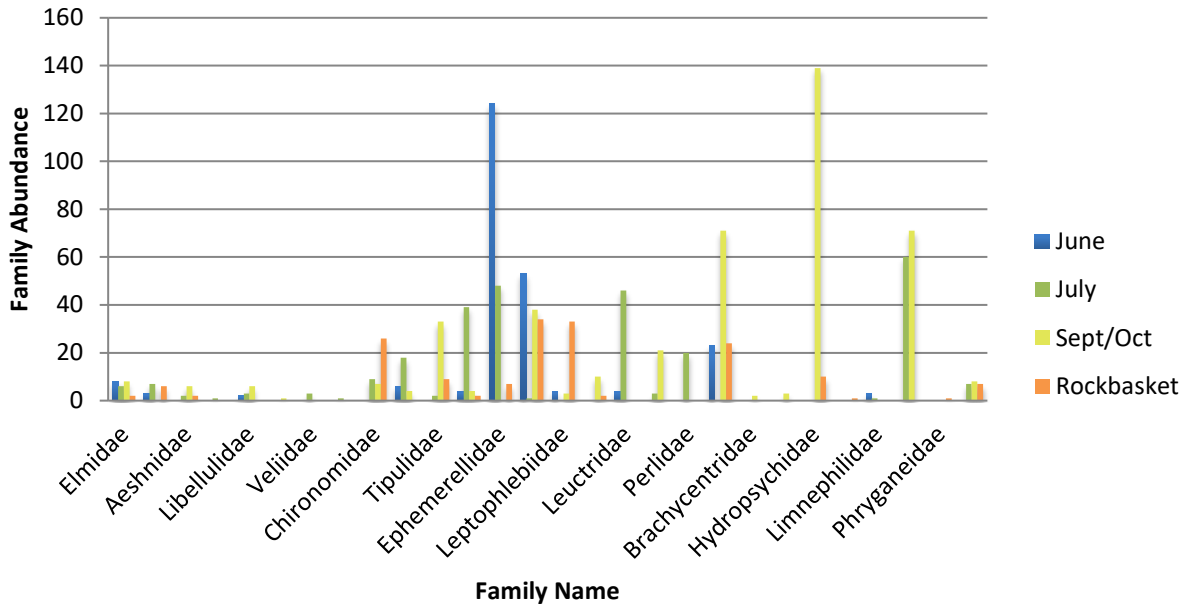
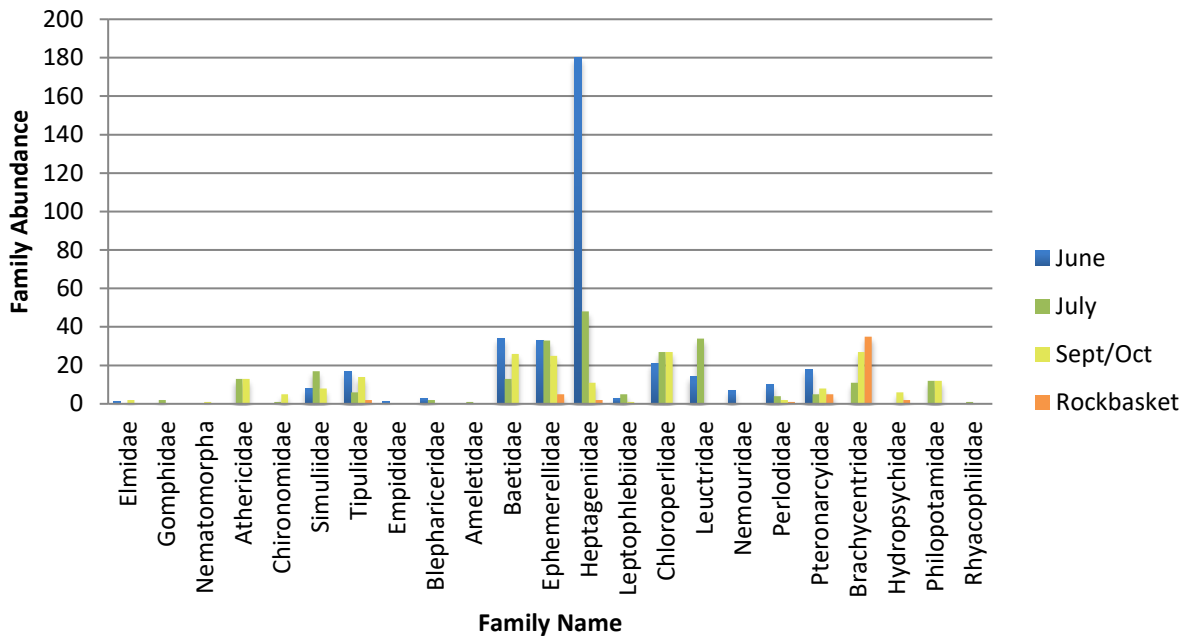**Clay Brook Macroinvertebrate Family Abundance 2017**
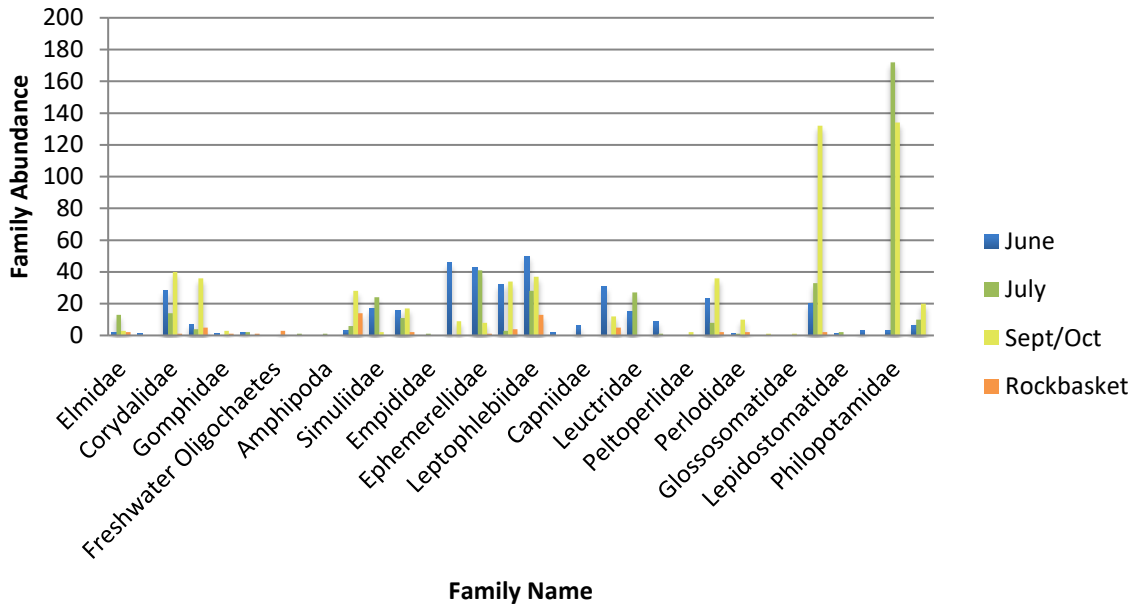
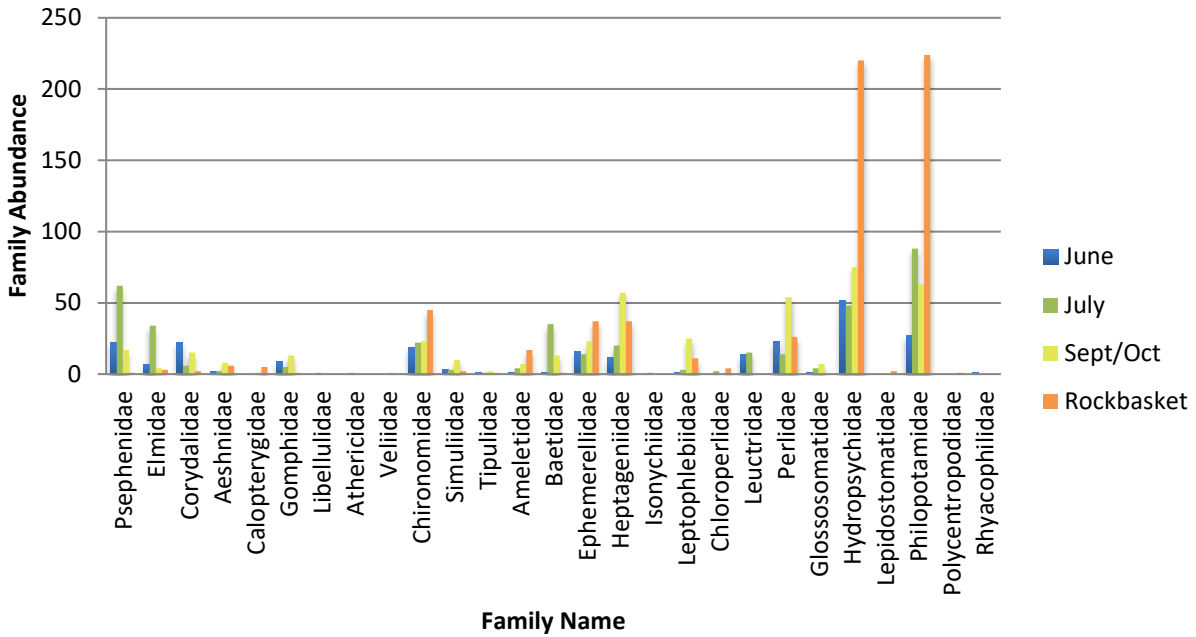**Cockermouth River Macroinvertebrate Family Abundance 2017**

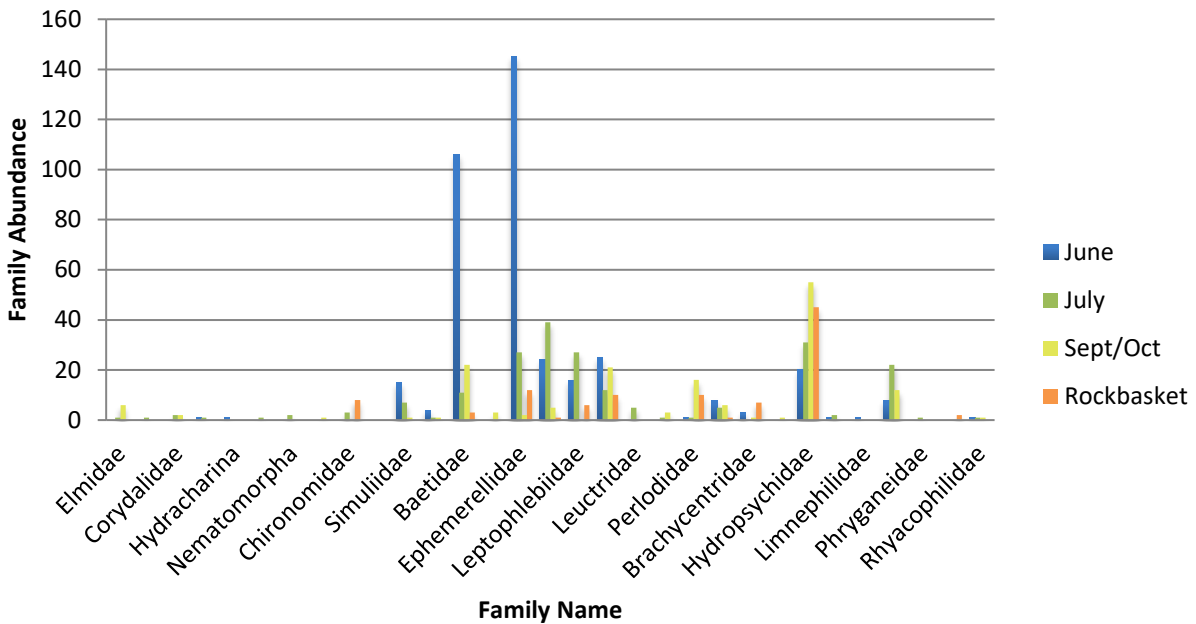Halfway Brook Macroinvertebrate Family Abundance 2017
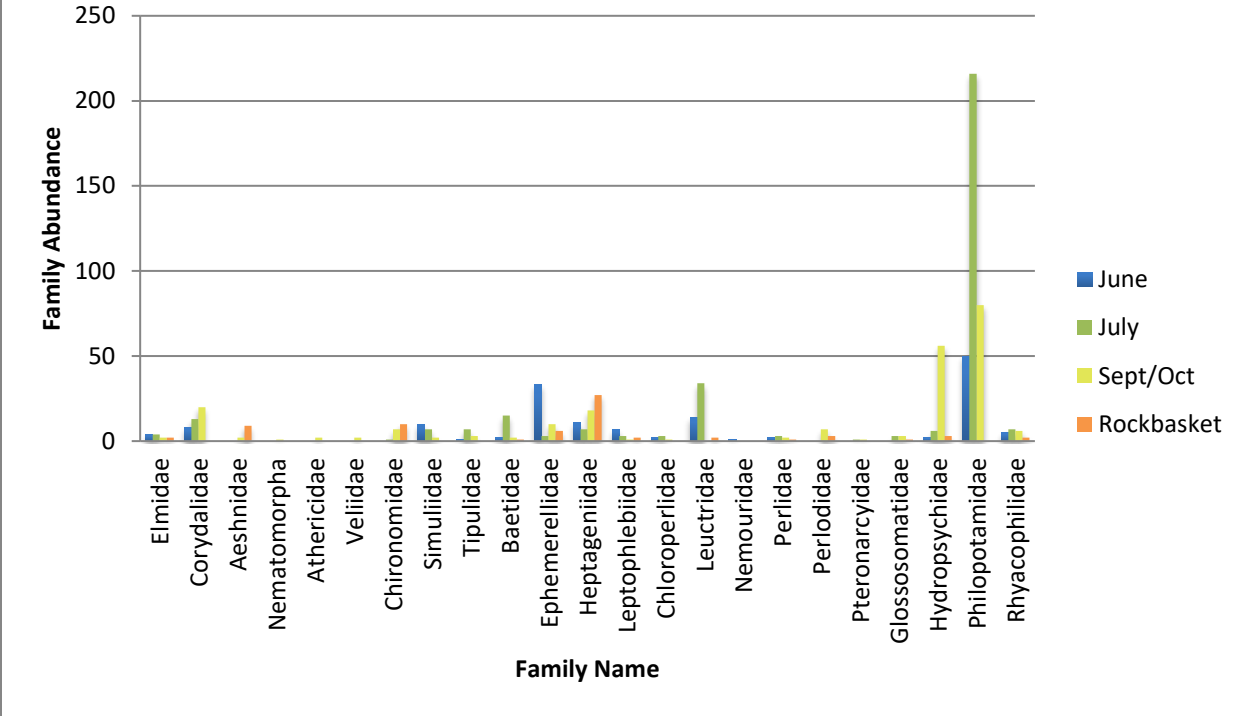

Mad River Macroinvertebrate Family Abundance 2017

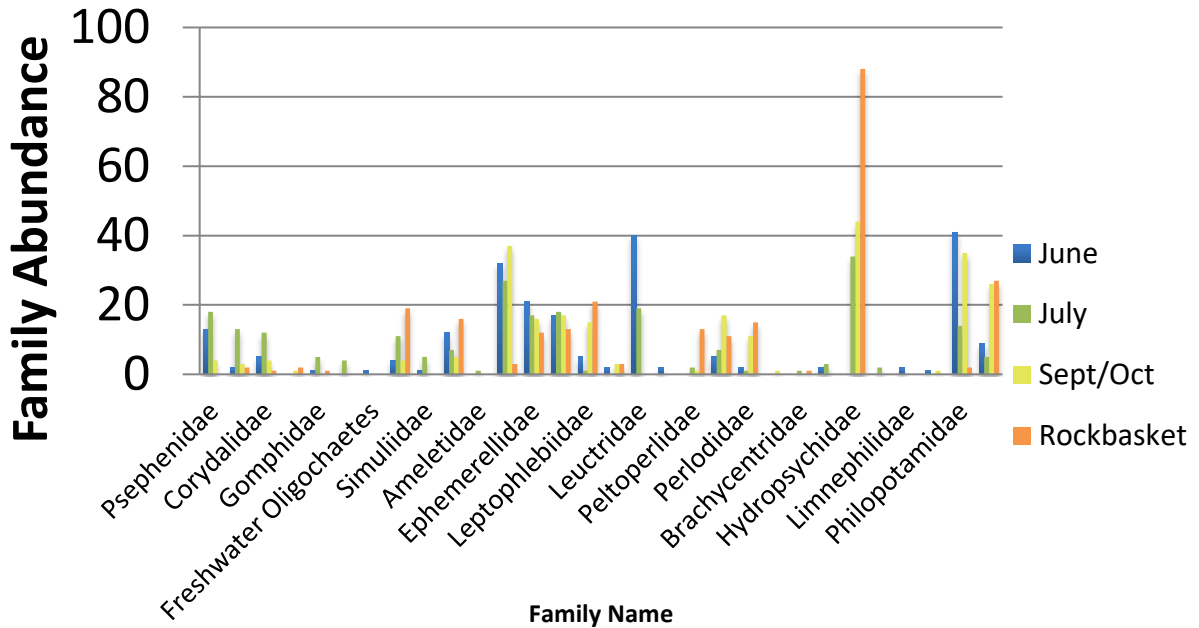**Otter Brook Macroinvertebrate Family Abundance 2017**

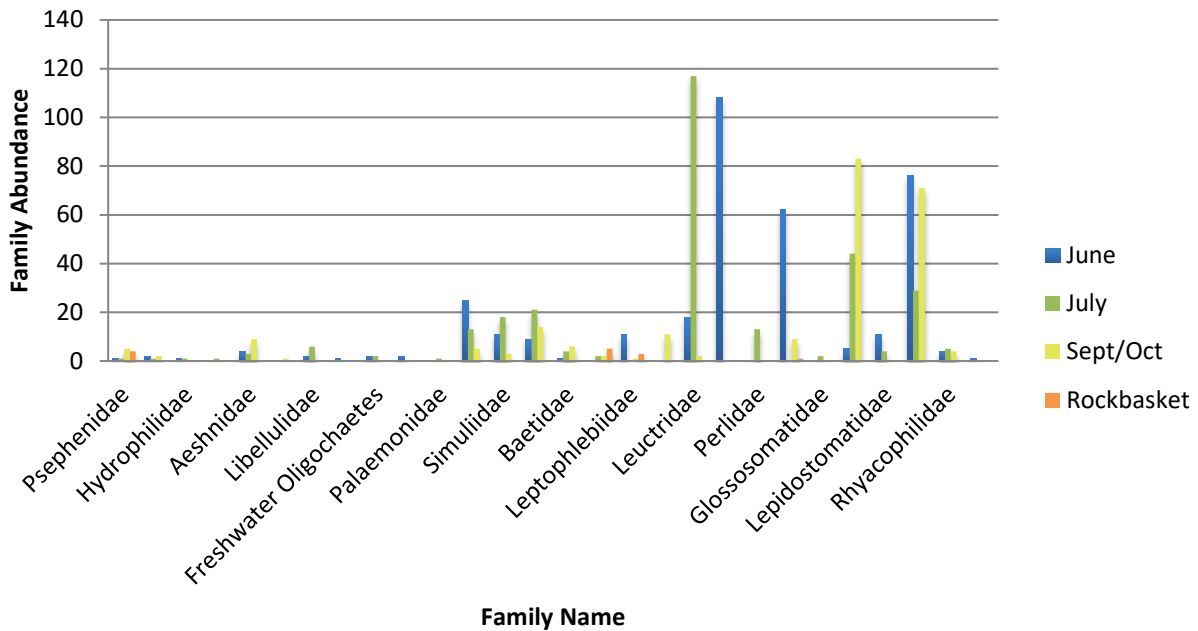**Pemigewasset River Macroinvertebrate Family Abundance 2017**

Shannon Brook Macroinvertebrate Family Abundance 2017

Sucker Brook Macroinvertebrate Family Abundance 2017

Wednesday Hill Brook Macroinvertebrate Family Abundance 2017

Appendix B: R-squared values of univariate relationships between chloride and benthic macroinvertebrate metrics from 2017. Values with * are significant (alpha = 0.05), blue reflects a positive and green text a negative relationship with chloride observed.

Response Variable	June-Oct	June	July	Sept-Oct	Rockbaskets
Total Macroinvertebrates	0.0098	0	0.0001	0.0751	0.002
% EPT	0.0043	0.0183	0.0214	0.026	0.003
% Ephemeroptera	0.2863*	0.3406	0.352	0.3053	0.3912
% Plecoptera	0.2206*	0.514*	0.4477*	0.0006	0.2876
% Tricoptera	0.0977	0.1159	0.0132	0.2753	0.0349
% Chironomidae	0.076	0.3276	0.0695	0.0387	0.2161
% Diptera	0.0537	0.1383	0.2483	0.0078	0.362
Total Family Richness	0	0.0154	0.0001	0.1819	0.1514
EPT Family Richness	0.1414*	0.0644	0.3058	0.2209	0.1475
Ephemeroptera Richness	0.2246*	0.2942	0.2292	0.2407	0.0113
Plecoptera Richness	0.2408*	0.1949	0.3903	0.2028	0.4617*
Tricoptera Richness	0.0549	0.0919	0.0676	0.0164	0.0663
Chloroperlidae Relative Abundance [IT]	0.0523	0.1968	0.0964	0.0213	0.1827
Leuctridae Relative Abundance [IT]	0.0539	0.0005	0.6579*	0.3129	0.039
Philopotamidae Relative Abundance [IT]	0.0217	0.1268	0.002	0.1003	0.0076
Rhyacophilidae Relative Abundance [IT]	0.0212	0.0362	0.0008	0.1037	0.0433
Simuliidae Relative Abundance [T]	0.0018	0.0175	0.0318	0.0023	0.0086

* = significant

positive

negative