Ecotoxological responses of stream mayflies exposed to elevated chloride in source waters that differ in hardness

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Executive Summary

Recent analyses have found that chloride concentrations in surface waters have been increasing over the last several decades, at multiple locations. Sources contributing to the increase in chloride concentration include road runoff following applications of deicing products, effluents from wastewater treatment plants (e.g., reflecting use of water softeners, salt in the human diet), wastewaters from some industrial and oil and gas production activities, and runoff and groundwater associated with various agricultural practices. At times, it appears that ambient chloride concentrations in streams and rivers reach levels may have a negative affect on aquatic organisms.

The ecotoxicity study described in this report quantified acute responses of six mayfly species in short-term exposures to Cl, and chronic responses of four mayfly species in full life-cycle exposures to Cl (delivered in the form of NaCl) in water that ranged from soft (<10 mg/L) to hard (>200 mg/L). Mayflies are common and important components of stream ecosystems, and are known to be relatively sensitive to changes in water quality and play an important role in the commonly used metrics used to assess stream health. The presence or conspicuous absence of certain mayfly species at a site is a meaningful record of environmental conditions during the recent past, including ephemeral events that might be missed by assessment programs that rely on periodic water chemistry samples. Water hardness has been known to affect chloride toxicity for some aquatic organisms, but nothing is known about how hardness affects chloride toxicity for mayflies.

The acute (48 h) toxicity tests were run with four source waters and newly hatched 1st instar for six mayfly species representing four different families: Baetidae *Neocloeon triangulifer*, *Anafroptilum semirufum*, *Procloeon fragile,* Ephemerellidae *Ephemerella invaria*, Leptophelbiidae *Leptophlebia cupida*, and Heptageniidae *Macaffertium modestum*.

- Based on acute LC50s (concentrations that were lethal to 50% of the individuals in test), three of six mayfly species (i.e., the baetid mayflies *N. triangulifer*, *A. semirufum*, *P. fragile*) are relatively sensitive (e.g., LC50s = 1077-1885 mg Cl/L in moderately hard water) to chloride in a brief (48 h) exposure compared with the other three species (i.e., *E. invaria*, *L. cupida*, *M. modestum*; e.g., LC50s = 3433-5194 mg Cl/L in moderately hard water).
- Acute chloride toxicity for five of six mayflies examined (*N. triangulifer*, *A. semirufum*, *P. fragile*, *E. invaria*, *M. modestum*, but not *L. cupida*) is greater when the test waters are soft relative to moderately hard and/or hard waters. For example, LC50s for *P. fragile* were 462-472 mg Cl/L in soft water versus 1661-2263 mg Cl/L in moderately hard and hard waters.
- Based on LC50s from experiments with moderately hard water, these six mayfly species are not the most sensitive aquatic species when exposed to elevated chloride for 48 h.

However, this result should be interpreted with caution as data for many other species represent 96 h exposures.

- Acute LC50s for baetid mayflies in soft water were among the lowest known for aquatic organisms.

Our chronic (full life cycle) experiments involving three different mayfly species (all from a single mayfly family, Baetidae) resulted in six general observations

- Among the chronic response variables examined (i.e., survivorship, development time, adult body size, instantaneous growth rate, and population growth rate), survivorship (i.e., LC20: concentrations that were lethal to 20% of the individuals in test) was consistently the most sensitive response variable for chloride (population growth rate was a close second, because it primarily reflects survivorship in chloride exposures).
- All three mayfly species (i.e., *N. triangulifer*, *A. semirufum*, *P. fragile*) are relatively sensitive to chloride in a long-term (22-43 d) exposure to chloride (e.g., LC20s = 175-332 mg Cl/L in moderately hard water).
- Chronic chloride toxicity for the mayflies examined is greater when the test waters are soft relative to moderately hard and/or hard waters. For example, LC50s for *N. triangulifer*, *A. semirufum*, and *P. fragile* were 109, 114, and 168 mg Cl/L, respectively, in soft water versus 175, 279, and 332 mg Cl/L, respectively, in moderately hard waters
- A fourth species, *M. modestum*, was exposed to chloride of 50 d (part of a life cycle) in moderately hard water, and survivorship after 50 d shows that *M. modestum* is also relatively sensitive to chloride (chronic LC20 = 138 mg Cl/L), similar to the three baetid species and in contrast to the acute results for *M. modestum*, which was moderately tolerant (acute LC50 = 3433-3718 mg Cl/L). This suggests that moderate tolerance to an acute exposure to chloride may not translate into a moderate tolerance to a chronic exposure to chloride for some mayflies.
- In moderately hard water, these four mayfly species are sensitive to exposure to elevated chloride, but were not the most sensitive aquatic species.
- When the three baetid species were exposed to chloride in soft water, the chronic LC20s were among the lowest chronic values known.

All of the above toxicity results are from experiments that began with 1st instar mayflies. Thus, the data represent the more sensitive life stages for an important portion of the stream macroinvertebrate fauna used in stream assessments. A comparison of selected water quality criteria with the toxicity results from this study highlight three issues for the established criteria.

- The results demonstrate that hardness can play an important role in chloride toxicity for mayflies, and most criteria do not account for differences in hardness among streams.
- Acute criteria are often lower than the relevant LC50s for mayflies, but this may not be true for some species in soft water (e.g., *A. semirufum* or *P. fragile*).
- Chronic criteria >200 mg Cl/L could be associated with measurable mayfly mortality (based on LC20s), especially in soft waters.

Introduction

The ion composition of natural waters reflects three primary mechanisms: atmospheric precipitation, rock dominance, and evaporation-crystallization processes (Gibbs 1970). It is these processes, working together or separately, that have resulted in surface waters that range widely in both ionic strength (e.g., salinity, total dissolved solids, or specific conductance) and composition (i.e., concentrations of Na, Ca, Mg, K, CO_3, SO_4, Cl). The ionic composition of natural stream waters in most regions of the United States is predominately Ca Ca, Mg, and CO_3 (Griffith 2014). Increased dominance of SO_4 or Cl was observed in some eastern US regions. This change appears to reflect atmospheric contributions of SO_4 or Cl to low ionic strength waters. In the case of SO_4, this contribution appears to be associated with acid rain (although local mining could also be a contributor). In contrast, Cl is more dominant in areas where marine air masses are important contributors of ions to surface waters (Griffith 2014).

Recent analyses have found that chloride concentrations in surface waters have been increasing over the last decades, at multiple locations (Goodwin et al. 2003, Interlandi and Crockett 2003, Kaushal, et al. 2005, Kelly et al. 2008). Sources contributing to the increase in chloride concentration include road runoff following applications of deicing products (Granato 1996, Corsi et al. 2010, Kelting et al. 2012), effluents from wastewater treatment plants (e.g., reflecting use of water softeners, salt in the human diet, e.g., Kelly et al. 2010, 2012), wastewaters from some industrial and oil and gas production activities (e.g., Hayes 2009, Kelly et al. 2010, Haluszczak et al. 2013), and runoff and groundwater associated with various agricultural practices. At times, it appears that ambient chloride concentrations reach levels may have a negative affect on aquatic organisms (Corsi et al. 2010, Gardner and Royer 2010, Bartlett et al. 2012a, b, Allert et al. 2012, Todd and Kaltenecker 2012, Porter-Goff et al. 2013).

The study described in this report was designed to provide some information needed to help set regulatory standards for chloride that are protective across the range of aquatic habitats and species found in Pennsylvania. We address four issues that could affect the development of widely applicable standards. First, it has been known for over 25 years that chloride toxicity is affected by concentrations of other common anions and cations in solution (especially calcium, e.g., Mount et al. 1997, Soucek et al. 2011), and the ionic composition of freshwaters can differ greatly across Pennsylvania (Griffith 2014). Second, much of the currently available bioassay data reflect studies of well known, commonly used standard laboratory animals such as the water flea *Ceriodaphnia dubia* and the fathead minnow *Pimephales promelas*, and little is known about how these results relate to chronic chloride exposure for the diverse floras and faunas that inhabit the freshwaters of Pennsylvania. Third, there is growing evidence that some life stages can be more sensitive to environmental stressors relative to other life stages (e.g., younger versus older aquatic insect larvae; Gauss et al. 1985, Williams et al. 1986, Kiffney and Clements 1996, Clark and Clements 2006), yet many toxicity studies have failed to include the more sensitive life stages (Clements et al. 2013). Finally, acute toxicity data for chloride is more common than chronic data (Stroud Water Research Center 2010, CCME 2011, Elphick et al. 2011), yet chronic exposures are vital given that field data show chloride concentrations are elevated even at base flow.

Benthic macroinvertebrates were chosen to be the subject of this ecotoxicity study of chloride because benthic macrionvertebrates are an ecologically important group of aquatic organisms that is commonly included in water quality assessment programs (Hellawell 1986, Resh 2008), and because they have provided water quality assessment programs with valuable insight for more than 100 years (Cairns and Pratt 1993). The presence or conspicuous absence of certain macroinvertebrate species at a site is a meaningful record of environmental conditions during the recent past, including ephemeral events that might be missed by assessment programs that rely on periodic water chemistry samples (Weber 1973, Barbour et al. 1999). Among the aquatic macroinvertebrates, we focused on mayflies because they are known to be relatively sensitive to changes in water quality and play an important role in the commonly used EPT Index or EPT Richness (Lenat and Penrose 1996) as well as other metrics that are common components of biomonitoring multimetrics used in standard water quality assessment protocols (e.g., Total Richness, EPT Richness, Beck's Index, Hilsenhoff Biotic Index, Percent Sensitive Individuals in PA IBI; PADEP 2013).

The experiments described here were designed to provide insights into an important environmental protection and management question – how vulnerable are stream biota to elevated concentrations of chloride? Previous research on standard laboratory animals indicates that vulnerability could depend on the species examined, and the chemistry of the waterway receiving the chloride (i.e., water chemistry characteristics such as calcium or magnesium concentration can affect chloride toxicity). We examine this question through a series of acute and chronic bioassays using 3-6 mayfly species that are native to Pennsylvania and distributed throughout eastern North America, and that we have now developed methods for laboratory rearing and experimentation. As part of these bioassays, we varied water chemistry by using water from 3-4 streams that varied in hardness from soft (<10 mg/L) to hard (>200 mg/L).

Experimental Design and Methods

Source Waters

Water from three source-water streams was collected on three dates (Table 1, Appendix 1). The soft-water stream was Spruce Run (41° 1'26.19"N, 77° 3'55.03"W) in Union Co. PA. The moderately hard-water stream was an unnamed tributary to House Run (39°51'48.28"N, 80°18'49.69"W) in Greene County PA. The hard-water stream was Cedar Run (41° 0'28.59"N, 77° 8'53.87"W) in Union Co. PA. Reference water from each experiment was collected from White Clay Creek (39°51'38.41"N, 75°47'01.96"W), Chester Co. PA, which was also moderately hard and was where all of the study species were originally collected.

Table 1. Chemical characteristics of source waters – values from 2 Jul 2014 collection date (see Appendix 1 for complete data).

	Low	Medium	High	Medium Reference
	Spruce	House	Cedar	WCC
pH	6.9	8.1	8.5	7.8
Conductivity (µS/cm)	18.1	189.4	434	230
Hardness (mg CO_3/L)	7	86	218	91
Ca^{2+} (mg/L)	1.5	26.8	53.6	22.4
Mg^{2+} (mg/L)	0.8	4.7	20.5	8.5
CO_3^{2-} (mg/L)	3.6	83.4	188.4	61.8
SO_4^{2-} (mg/L)	3.4	14.4	16.6	17.4
Cl^- (mg/L)	0.6	1.2	12.2	11.2
Total Dissolved Solids (mg/L)	20	142	274	166 *

* This value for TDS is from a WCC sample collected 3 Sep 2014.

Study Species

We quantified acute responses of six mayfly species in short-term exposures to Cl, and chronic responses of four mayfly species in full life-cycle exposures to Cl delivered in the form of NaCl (Table 2).

Table 2. List of mayfly species used in acute and lifecycle (chronic) exposures to chloride. Experimental design details are in Appendixes 2 and 3. Pollution-tolerance values (TV) that would be assigned to these species (either based on species or genus) provided from Pennsylvania and EPA reference lists. Note that *Neocloeon triangulifer* and *Anafroptilum semirufum* were listed as *Centroptilum* until Jacobus and Wiersema (2014), and the tolerance values presented here are for *Centroptilum*.

Family	**Species**	**Acute**	**Lifecycle**	**PATV**[1]	**EPATV**[2, 3]
Baetidae	*Neocloeon triangulifer*	X	X	2	2.5
	Anafroptilum semirufum	X	X	2	2.5
	Procloeon fragile	X	X	6	2.8
Ephemerellidae	*Ephemerella invaria*	X		1	3.0
Leptophelbiidae	*Leptophlebia cupida*	X		4	3.7
Heptageniidae	*Macaffertium modestum*	X	X	3	3.8

[1] Pennsylvania Department of Environmental Protection (2009)

[2] Unpublished data obtained in 2005 from US EPA

[3] Species specific values listed for *Ephemerella invaria* and *Macaffertium modestum*.

Baetidae: *Neocloeon triangulifer* (McDunnough, 1931)

Neocloeon triangulifer was until recently classified as a species of *Centroptilum* (Jacobus and Wiersema 2014) and before that as a species of *Cloeon* (McCafferty and Waltz 1990). It is a parthenogenetic (clonal) mayfly species (Sweeney and Vannote 1984, Funk et al. 2006) that is most abundant during summers when it has a relatively rapid development (25-30 d at 20°C). The primary clone we have worked with in these studies (i.e., Stroud Water Research Center [SWRC] Clone WCC-2®) occurs in low numbers during the winter, with minimal growth below 10°C. The WCC-2 clone was initially obtained from White Clay Creek, a piedmont stream of moderately high hardness located in southeastern PA. This stream has been designated Exceptional Value or defined as the "best" cold-water fisheries by PA Department of Environmental Protection. We find *N. triangulifer* commonly in the along the edges and slow current areas of 3rd – 4th order streams in the mid-Atlantic region of the United States. Literature and online sources plus our own collections include records from 19 states and provinces ranging from Quebec to Florida in the east and from Iowa to Texas in the west. This species been used for toxicity studies by Stroud since the early 1990s (e.g., Sweeney et al, 1993). Parthenogenetic reproduction (Funk et al. 2006) has allowed us to maintain cultures of a specific clone (WCC-2) of *N. triangulifer* in the laboratory almost continuously since the late 1980s, without risk of inbreeding or other genetic changes. This specific clone has also been recently used in a number of experiments examining the effects of thermal changes and extremes (SWRC unpublished data), elevated phosphorus (SWRC unpublished data), and the toxic effects of cadmium, mercury, selenium and zinc (Conley et al. 2009; Xie et al. 2009; Xie et al. 2010; Conley et al. 2011, Xie and Buchwalter 2011; Kim et al. 2012) and various ions in solution (Soucek and Dickinson, In Press; David B. Buchwalter, NC State University, unpublished data; James Lazorchak, USEPA Cincinnati, personal communication).

Baetidae: *Anafroptilum semirufum* (McDunnough, 1926)

Anafroptilum semirufum was until recently was classified as a species of *Centroptilum* (Jacobus and Wiersema 2014). It is a sexual mayfly species that exhibits a life history similar to that of *N. triangulifer* except that it has a winter egg diapause. It is most abundant during summers when it has a relatively rapid development (25-30 d at 20°C). We find it along the edges and slow current areas of 2nd – 4th order streams in the mid-Atlantic region of the United States. Literature and online sources plus our own collections include records from four states and provinces ranging from Ontario to North Carolina. We have been working with *A. semirufum* in the laboratory since 2004, collecting larvae from White Clay Creek as needed. We have not maintained a culture in the laboratory because of the risk of inbreeding and other genetic changes.

Baetidae: *Procloeon fragile* (McDunnough, 1923)

Procloeon fragile was originally classified as a species of *Centroptilum*, but was later reclassified as a species of *Procloeon* (McCafferty and Waltz 1990). It is a sexual mayfly species that exhibits a life history similar to that of *N. triangulifer* except that it has a winter egg diapause. It is most abundant during summers when it has a relatively rapid development (25-30 d at 20°C). We find it along the edges and slow current areas of 3rd – 8th order streams in the mid-Atlantic region of the United States. Literature and online sources plus our own collections include records from seven states (including NY, PA) and provinces ranging from Ontario to Alabama. We have been working with *P. fragile* in the laboratory since 2004, collecting larvae from White Clay Creek as needed. We have not maintained a culture in the laboratory because of the risk of inbreeding and other genetic changes.

Ephemerellidae: *Ephemerella invaria* (Walker, 1853)

Ephemerella invaria is a predominantly sexual mayfly species (some parthenogenetic populations are known) common in streams throughout eastern North America. Literature and online sources plus our own collections include records from 17 states (including PA, NY) and provinces ranging from Quebec to Alabama in the east and from Minnesota to Arkansas in the west. Specimens in this study were from White Clay Creek, Chester Co., PA. The species exhibits a univoltine life history. Eggs laid in mid- to late-May in White Clay Creek and begin hatching in mid-July.

Heptageniidae: *Maccaffertium modestum* (Banks, 1910)

Maccaffertium modestum is a sexual mayfly species common in streams throughout eastern North America. Literature and online sources plus our own collections include records from 25 states (including PA, NY, OH, WV) and provinces ranging from Nova Scotia to Florida in the east and from Minnesota to Texas in the west. It exhibits a multivoltine life history with eggs hatching in 12 d at 20°C and larval development time is about 80 days. Specimens in this study were from White Clay Creek, Chester Co., PA

Leptophelbiidae: *Leptophlebia cupida* (Say 1923)

Leptophlebia cupida is a sexual mayfly species common in streams throughout eastern United States and Canada. Literature and online sources plus our own collections include records from 25 states (including PA, NY, OH, WV) and provinces ranging from Newfoundland to Alabama in the east and from British Columbia to Colorado in the west. It exhibits a univoltine life history that begins with eggs hatching in mid-June. Typically these will not emerge until the following April. However, we have reared hatchlings at constant 20° and larvae required 4 to 6 months to complete development. Specimens in this study were from White Clay Creek, Chester Co., PA

Experiment 1: *Mayfly Responses to Acute Exposures to Cl*

We quantified acute responses of six mayfly species (Table 2) in short-term exposures to Cl delivered in the form of NaCl. NaCl was chosen because Na is known to have little effect on the toxicity of Cl relative to other cations such as Ca (which can reduce Cl toxicity) or Mg and K (which can increase Cl toxicity) (Mount et al. 1997). We conducted 96 acute tests (each test had one replicate of 20 individuals each; Table 3, Appendix 2) that included four source waters (Spruce, House, Cedar, White Clay), with newly hatched 1st instar for *N. triangulifer*, *A. semirufum*, *P. fragile, E. invaria*, *L. cupida*, and *M. modestum*. Each test had six treatments: a control (0 mg Cl/L added in Spruce, House, Cedar and White Clay water) and five dilution concentrations (250, 500, 1000, 2000, 4000 mg Cl/L, or 500, 1000, 2000, 4000, 8000 mg Cl/L added to Spruce, House, Cedar and White Clay water, Table 3). These were static (no renewal) experiments, conducted at 20°C for 48 h. 20µL of a diatom slurry was provided as food in each test vessel.

Response assessed was survivorship reported as concentration of chloride associated with 50% mortality (lethal concentration associated with 50% mortality; LC50) of test population from that observed under control conditions. Comparable LC50 values were determined using the nonparametric trimmed-Spearman-Karber (tS-K) method (Harmen et al. 1977, EPA 2002) and Probit regression analysis (SAS STAT v.9.3). Both analyses used smoothed and control-adjusted survival data and employ log10-transformed treatment values. Data smoothing requires ordering the data by increasing treatment concentrations, taking the mean of percent survival values for those treatments having lower survivorship relative to subsequent, higher concentration, treatments. These smoothed percent survival values were then adjusted for control treatments having < 100% survival by dividing by the control-treatment survival percentage. Specific to the tS-K method, smoothed-adjusted survival values were 'trimmed' using a consistent value of 20%. Treatments with survival percentages < 20% or > 80% were removed and treatment values, corresponding to the 20% and 80% survival limits, were estimated via interpolation from the smoothed-adjusted values bracketing those two limits. The final LC50 value from the tS-K method is determined by summing the weighted mean of each pair of treatment values with the weights equal to the difference in survival percentages of the corresponding pair of treatments. The LC50 value from the Probit procedure is the treatment value at 50% survival predicted from the probit model of smooth-adjusted percent survival data versus log10-transformed treatment concentrations. Differences among source waters for acute LC50s were assessed with one-way analysis of variance followed by a Tukey's range test comparing source waters for each species.

Table 3. Chloride concentrations (mg Cl/L) used in acute (48-h) and lifecycle (chronic) exposure experiments.

	Ambient	Low		Hardness		High
Acute [1]	0	250	500	1000	2000	4000
Acute [2]	0	500	1000	2000	4000	8000
Lifecycle [1, 3]	0	125	250	500	1000	2000

[1] Baetidae *Neocloeon triangulifer*, *Anafroptilum semirufum*, and *Procloeon fragile*, and for lifecycle exposure Heptageniidae *Macaffertium modestum*

[2] Ephemerellidae *Ephemerella invaria*, Leptophelbiidae *Leptophlebia cupida*, Heptageniidae *Macaffertium modestum*

[3] A partial lifecycle test (50 d) was conducted for Heptageniidae *Macaffertium modestum*

Experiment 2: *Mayfly Responses to Chronic (whole lifecycle) Exposures to Cl*

Experiment 2 was designed to quantify chronic responses of *N. triangulifer*, *A. semirufum*, and *P. fragile* in whole lifecycle exposures to NaCl in three different sources waters, and compare these results to acute data for these species (Table 1). These were whole-life cycle tests that began with the introduction of 50 newly hatched, first-instar larvae into each 1.9-L jars filled with water (static, no renewal) and an air stone to maintain oxygen saturation. Larvae were fed algae grown on acrylic plates, replenished as needed. For each test there were 6 treatments (0, 206, 412, 824, 1647, and 3295 mg/L added NaCl, representing 0, 125, 350, 500, 1000, and 2000 mg Cl/L) with four replicate jars per treatment (Table 3, Appendix 3). Tests were run at 20°C until all remaining larvae had emerged as adults (i.e., 20-48 days). Emerging adults were trapped in cages over the jars and collected daily. An additional whole-life cycle test was run for each species with water from White Clay, but it was not replicated. Basic water chemistry data for these tests are presented in Appendix 1.

Chronic response variables that could be measured were survivorship (%), development time (days), final body size (mg dry mass), instantaneous growth rate (IGR), and population growth rate (PGR). IGR reflects the growth (biomass) that occurs per day from 1st instar to adult and is calculated as: $\ln(W_f/W_i)$/development time in days, where W_f = final adult dry mass in mg (gravid weight in the case of females) and W_i = 0.0009 mg (hatchling mass). PGR is expressed as the number of eggs produced per individual (present at the start of the experiment) per day, calculated as: (fecundity x survivorship)/development time. This value is halved for sexual species (assuming half the population is female). Hypothesis tests (ANOVA with Tukey post hoc test) were used to determine the No Observed Effect Concentration (NOEC, the highest concentration that did not differ from the control), and Lowest Observed Effect Concentration (LOEC or lowest test concentration that differed from control).

As in acute tests, comparable LC50 values were determined using the nonparametric trimmed-Spearman-Karber (tS-K) method (Harmen et al. 1977, EPA 2002) and Probit regression analysis (SAS STAT v.9.3). The LC50 value from the Probit procedure is the treatment value at 50% survival predicted from the probit model of smooth-adjusted percent survival data versus log10-transformed treatment concentrations. These Probit models were also used to estimate LC20 and LC10 values as well as EC20 (Effective Concentration - the concentration showing 20% effect on a variable) values for Population Growth Rate. EC20s for other non-lethal variables (i.e., development time, body size, Instantaneous Growth Rate) were estimated with linear regressions comparing the range of chloride concentrations with measurable responses (i.e., in the active portion of the dose response curve where larvae survived and adults emerged; extra zeros were not included).

For instantaneous growth and development time, the Spruce Run tests all experienced complete mortality at the 500 mg/L Cl treatment (and, in some tests, even at the 250 mg/L treatment level). In order to generate suitable relationships between these variables and Cl concentrations, a substitution routine was run to generate hypothetical growth or development times corresponding to the 500 mg/L treatment. Using available data from the other source-water experiments, mean growth and development time ratios were calculated for the 500 mg/L treatment value relative to the 125 or 250 mg/L treatment value. These ratios were then multiplied by the Spruce Run growth or emergence value for the 125 or 250 mg/L treatment value having survivors, leading to an estimate of growth or development time for the 500 mg/L treatment. The generation of substitute data for the 500 mg/L treatment allows the regressions to be more comparable among tests and waters. Differences among source waters for chronic LC20s and EC20s were assessed with one-way analysis of variance followed by a Tukey's range test comparing source waters.

Results

Experiment 1: *Mayfly Responses to Acute (48-h) Exposures to Cl*

The acute (48 h) toxicity tests were run with four source waters (Spruce, House, Cedar, White Clay) and newly hatched 1st instar for six mayfly species representing four different families: *N. triangulifer*, *A. semirufum*, *P. fragile, E. invaria*, *L. cupida*, and *M. modestum*. Results from the individual replicates were assessed separately. Only four replicates had <90% survivorship in the control (all were *N. triangulifer* in House water). The dose-response results in these replicates were otherwise good and they were included in our analyses. In addition, 29 of 96 replicates had a significant Goodness-of-Fit result for the Probit model these were examined individually to determine if the significant difference reflected a meaningful divergence from the expected Probit model shape. In all cases the estimated LC50s for those replicates were considered to be relatively similar to the others, thus none were omitted from the analyses.

Acute Survivorship (%)

Neocloeon triangulifer

Based on Spearman-Karber analyses, the LC50 for a 48-h exposure for *N. triangulifer* was 712 mg Cl/L in Spruce water, 2301 mg Cl/L in House water, 1551 mg Cl/L in Cedar water, and 1667 mg Cl/L in White Clay water. Based on Probit analyses, the LC50 for a 48-h exposure for *N. triangulifer* was 704 mg Cl/L in Spruce water, 2141 mg Cl/L in House water, 1420 mg Cl/L in Cedar water, and 1885 mg Cl/L in White Clay water. Both Spearman-Karber and Probit values were significantly lower from Spruce than from House and White Clay. Values from Cedar did not differ significantly from any of the other three water sources.

Anafroptilum semirufum

Survivorship was assessed for 1st instar *A. semirufum* in Spruce, House, Cedar, and White Clay waters. Similar to *N. triangulifer*, *A. semirufum* was more sensitive to elevated Cl concentrations in Spruce water relative to House, Cedar, or White Clay water (Table 4). Based on Spearman-Karber analyses, the LC50 for *A. semirufum* was 71 mg Cl/L in Spruce water versus 1826 mg Cl/L in House, 1354 mg Cl/L in Cedar, and 1235 mg Cl/L in White Clay water. Based on Probit analyses, the LC50 for *A. semirufum* was 107 mg Cl/L in Spruce water versus 1827 mg Cl/L in House, 1336 mg Cl/L in Cedar, and 1077 mg Cl/L in White Clay water. *A. semirufum* appears to be more sensitive than 1st instar *N. triangulifer* in acute exposures to elevated Cl concentrations in soft water (i.e., 71-107 versus 704-712 mg Cl/L in Spruce water), and somewhat more sensitive in moderately hard water (i.e., 1826-1827 versus 2141-2301 mg Cl/L in House water; 1077-1235 versus 1667-1885 mg Cl/L in White Clay water). However, the results for Cedar suggest that the difference between *A. semirufum* and *N. triangulifer* is not that great in acute exposures to elevated Cl concentrations in the hardest water we examined (i.e., 1336-1354 versus 1420-1551 mg Cl/L in Cedar water). Both Spearman-Karber and Probit values were significantly lower from Spruce than from House, White Clay, and Cedar. Thus, similar to *N. triangulifer*, there was increased sensitivity to Cl when hardness is low (e.g., Spruce versus the other source waters), but we saw little consistent difference for *A. semirufum* between our moderately hard (House, White Clay) and hardest water (Cedar).

Table 4. Acute (48 h) LC50 estimates (geometric mean with 95% Confidence Interval) for six mayfly species exposed to various chloride concentrations in four different source waters that differed in hardness (shown in parentheses).

	Spruce (6)		House (94)		Cedar (212)		WCC (89)	
Spearman-Karber	mean	95% CI	mean	95% CI	mean	95% CI	mean	95% CI
N. triangulifer	712	301-1688	2301	1572-3367	1551	985-2442	1667	890-3125
A. semirufum	71	17-307	1826	1350-2470	1354	686-2671	1235	441-3458
P. fragile	462	362-591	2263	1529-3350	2158	1955-2383	1883	852-4163
E. invaria	2077	1151-3747	5199	4810-5620	5473	5348-5600	5041	4674-5438
L. cupida	5304	4742-5933	5657	5341-5992	5586	5018-6218	5194	4259-6334
M. modestum	2067	1249-3420	3077	2094-4522	4533	3249-6324	3718	1999-6914
Probit								
N. triangulifer	704	319-1556	2141	1285-3568	1420	976-2067	1885	1133-3138
A. semirufum	107	25-452	1827	1145-2914	1336	748-2386	1077	435-2666
P. fragile	472	414-538	2110	1474-3019	1765	1590-1961	1661	725-3806
E. invaria	2016	1204-3375	4500	4053-4995	4762	4525-5011	4159	3703-4672
L. cupida	4667	3805-5724	5921	4843-7240	5832	3942-8628	4846	3841-6113
M. modestum	2065	1285-3319	2763	1886-4047	4329	2960-6330	3433	2109-5587

Procloeon fragile

Survivorship was assessed for 1st instar *P. fragile* in Spruce, House, Cedar, and White Clay waters. Similar to *N. triangulifer* and *A. semirufum*, *P. fragile* was more sensitive to elevated Cl concentrations in Spruce water relative to House, Cedar, or White Clay water (Table 4). Based on Spearman-Karber analyses, the acute LC50 for *P. fragile* was 462 mg Cl/L in Spruce water versus 2263 mg Cl/L in House water, 2158 mg Cl/L in Cedar water, and 1883 mg Cl/L in White Clay water. Based on Probit analyses, the acute LC50 for *P. fragile* was 472 mg Cl/L in Spruce water versus 2110 mg Cl/L in House water, 1765 mg Cl/L in Cedar water, and 1661 mg Cl/L in White Clay water. The results for Spruce water suggest that *P. fragile* may be intermediate between the more sensitive *A. semirufum* (i.e., 462-472 versus 71-107 mg Cl/L for *A. semirufum* in Spruce water) and the more tolerant *N. triangulifer* (i.e., 462-472 versus 704-712 mg Cl/L for *N. triangulifer* in Spruce water) in acute exposures to elevated Cl concentrations. However, the results for House, Cedar and White Clay suggest that *P. fragile* is similar to or less sensitive than either *N. triangulifer* or *A. semirufum* in acute exposures to elevated Cl concentrations (i.e., 2110-2263 versus 2141-2301 mg Cl/L for *N. triangulifer* and 1826-1827 mg Cl/L for *A. semirufum* in House water; 1765-2158 versus 1420-1551 mg Cl/L for *N. triangulifer* and 1336-1354 mg Cl/L for *A. semirufum* in Cedar water, 1661-1883 versus 1667-1885 mg Cl/L for *N. triangulifer* and 1077-1235 mg Cl/L for *A. semirufum* in White Clay water). Both Spearman-Karber and Probit values were significantly lower from Spruce than from House, White Clay, and Cedar. Thus, similar to *N. triangulifer* and *A. semirufum*, there is increased sensitivity to Cl for *P. fragile* when hardness is low (e.g., Spruce versus the other source waters), but we saw little consistent difference between our moderately hard (House, White Clay) and hardest water (Cedar).

Ephemerella invaria

Survivorship was assessed for 1st instar *E. invaria* in Spruce, House, Cedar, and White Clay waters. Similar to than *N. triangulifer A. semirufum*, and *P. fragile*, *E. invaria* was more sensitive to elevated Cl concentrations in Spruce water relative to House, Cedar, or White Clay water (Table 5). Based on Spearman-Karber analyses, the acute LC50 for *E. invaria* was 2077 mg Cl/L in Spruce water versus 5199 mg Cl/L in House water, 5473 mg Cl/L in Cedar water, and 5041 mg Cl/L in White Clay water. Based on Probit analyses, the acute LC50 for *E. invaria* was 2016 mg Cl/L in Spruce water versus 4500 mg Cl/L in House water, 4762 mg Cl/L in Cedar water, and 4159 mg Cl/L in White Clay water. However, across all source waters, *E. invaria* was more tolerant of an acute exposure to elevated Cl than *N. triangulifer A. semirufum*, and *P. fragile*, with LC50s that were 2 or more times greater than were observed for the three baetid species. For example, the LC50 for *E. invaria* in Spruce water was 2016-2067 mg Cl/L versus 71-712 mg Cl/L for *N. triangulifer A. semirufum*, and *P. fragile* in Spruce water. Similarly, the LC50 for *E. invaria* in Cedar water was 4762-5473 mg Cl/L versus 1336-2158 mg Cl/L for *N. triangulifer A. semirufum*, and *P. fragile* in Cedar water. Both Spearman-Karber and Probit values from Spruce were significantly lower than from House, White Clay, and Cedar. Thus, even though it was more tolerant to a short-term exposure to Cl, *E. invaria* again exhibited increased sensitivity to Cl when hardness is low (e.g., Spruce versus the other source waters) as with *N. triangulifer*, *A. semirufum*, and *P. fragile*. We saw little difference between our moderately hard (i.e., 4500-5199 mg Cl/L for House, 4159-5041 mg Cl/L for White Clay) and hardest water (4762-5473 mg Cl/L for Cedar).

Leptophlebia cupida

Survivorship was assessed for 1st instar *L. cupida* in Spruce, House, Cedar, and White Clay waters. Unlike the other five mayfly species examined, *L. cupida* did not exhibit marked differences in sensitivity to elevated Cl concentrations across the source waters (Table 4). Based on Spearman-Karber analyses, the acute LC50 for *L. cupida* was 5304 mg Cl/L in Spruce water versus 5657 mg Cl/L in House water, 5586 mg Cl/L in Cedar water, and 5194 mg Cl/L in White Clay water. Based on Probit analyses, the acute LC50 for *L. cupida* was 4667 mg Cl/L in Spruce water versus 5921 mg Cl/L in House water, 5832 mg Cl/L in Cedar water, and 4846 mg Cl/L in White Clay water. Across all source waters, *L. cupida* was the most tolerant of an acute exposure to elevated Cl of the mayfly species we examined, with LC50s that were 2-5 times greater for *L. cupida* than were observed for the three baetid species. For example, the LC50 for *L. cupida* in Spruce water was 4667-5304 mg Cl/L versus 71-712 mg Cl/L for *N. triangulifer A. semirufum*, and *P. fragile* in Spruce water. Similarly, the LC50 for *L. cupida* in Cedar water was 5586-5832 mg Cl/L versus 1336-2158 mg Cl/L for *N. triangulifer A. semirufum*, and *P. fragile* in Cedar water. Unlike *N. triangulifer*, *A. semirufum*, *P. fragile*, and *E. invaria*, both Spearman-Karber and Probit values from Spruce did not differ significantly than from House, White Clay, and Cedar. Thus, there was no clear increased sensitivity to Cl for *L. cupida* when hardness is low. Rather, we saw a relatively limited difference between our soft (Spruce), moderately hard (House, White Clay) and hardest water (Cedar). Overall, *L. cupida* was clearly more tolerant of an acute exposure to elevated Cl than *N. triangulifer*, *A. semirufum*, and *P. fragile*, and equal to or more tolerant than *E. invaria* (and *Macaffertium modestum*, see below).

Macaffertium modestum

Survivorship was assessed for 1st instar *M. modestum* in Spruce, House, Cedar, and White Clay waters. Similar to *N. triangulifer A. semirufum*, *P. fragile*, and *E. invaria*, *M. modestum* was more sensitive to elevated Cl concentrations in Spruce water relative to House, Cedar, or White Clay water (Table 4). Based on Spearman-Karber analyses, the LC50 for *M. modestum* was 2067 mg Cl/L in Spruce water versus 3077 mg Cl/L in House water, 4533 mg Cl/L in Cedar water, and 3718 mg Cl/L in White Clay water. Based on Probit analyses, the LC50 for *M. modestum* was 2065 mg Cl/L in Spruce water versus 2763 mg Cl/L in House water, 4329 mg Cl/L in Cedar water, and 3433 mg Cl/L in White Clay water. However, across all source waters, *M. modestum* was more tolerant of an acute exposure to elevated Cl than *N. triangulifer A. semirufum*, and *P. fragile*, with LC50s that were 30 to >100% more than were observed for the three baetid species. For example, the LC50 for *M. modestum* in Spruce water was 2065-2067 mg Cl/L versus 71-712 mg Cl/L for *N. triangulifer A. semirufum*, and *P. fragile* in Spruce water. Similarly, the LC50 for *M. modestum* in Cedar water was 4329-4533 mg Cl/L versus 1336-2158 mg Cl/L for *N. triangulifer A. semirufum*, and *P. fragile* in Cedar water. Both Spearman-Karber and Probit values from Spruce were significantly lower than from Cedar, but did not differ from House or White Clay. In addition, Cedar did not differ significantly for either House or White Clay. Thus, as with *N. triangulifer*, *A. semirufum*, *P. fragile*, and *E. invaria*, *M. modestum* exhibited a somewhat increased sensitivity to Cl for when hardness is low versus high (i.e., Spruce versus

Cedar water), but sensitivity in our moderately hard water (House, White Clay) could not be differentiated from either the softest (Spruce) or hardest water (Cedar). Overall, *M. modestum* was more tolerant of an acute exposure to elevated Cl than *N. triangulifer*, *A. semirufum*, or *P. fragile*, but comparable or less tolerant than *E. invaria* and *L. cupida*.

Experiment 2: *Mayfly Responses to Chronic (whole lifecycle) Exposures to Cl*

The chronic (whole lifecycle) test with chloride delivered as NaCl was conducted beginning with 1st instar larvae of *N. triangulifer*, *A. semirufum*, and *P. fragile* in four source waters (Spruce, House, Cedar, and White Clay). Analyses were conducted based on four replicates for Spruce, House, and Cedar, but only one replicate for White Clay. Development time (d), adult body size (mg), and Instantaneous Growth Rate (IGR, d^{-1}) for *A. semirufum* and *P. fragile* were calculated for males and females separately. Spearman-Karber and Probit methods were used to estimate LC50 for survivorship, and Probit was used to estimate LC20 and LC10 for survivorship and EC20 for Population Growth Rate (PGR). Simple linear regression (in the active portion of the dose response curve; extra zeros were not included) was used to estimate EC20 for development time and IGR.

We were also able to examine chronic survivorship for *Macaffertium modestum*, a heptageniid mayfly distantly related to the baetid mayflies that were the primary focus of the lifecycle tests. The development time for a complete life cycle of *M. modestum* is 60-80 days, double or more than that of *N. triangulifer*, *A. semirufum*, and *P. fragile*. Unfortunately, we had only enough individuals and water for two replicates, only in White Clay water, and only for 50 days (i.e., the experiment was ended before adult emergence).

Survivorship (%)

Neocloeon triangulifer

The no-observed-effect concentration (NOEC) for survivorship of *N. triangulifer* was 125 mg Cl/L in Spruce water, 500 mg Cl/L in House water, and 250 mg Cl/L in Cedar water (Figures 1 and 2). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for survivorship of *N. triangulifer* appears to be 500 mg Cl/L in White Clay water (Figure 1). Survivorship dropped in each of the next higher treatments. Thus, the lowest-observed-effect concentration (LOEC) for survivorship of *N. triangulifer* was 250 mg Cl/L in Spruce water (2% survivorship), 1000 mg Cl/L in House water (0% survivorship), and 500 mg Cl/L in Cedar water (23.5% survivorship) (Figures 1 and 2). The "non-statistical" LOEC for survivorship of *N. triangulifer* was 1000 mg Cl/L in White Clay water (Figure 1).

Based on Spearman-Karber analyses, the chronic LC50 for *N. triangulifer* was 103 mg Cl/L in Spruce water, 196 mg Cl/L in House water, and 365 mg Cl/L in Cedar water (Table 5). Based on Probit analyses, the chronic LC50 for *N. triangulifer* was 143 mg Cl/L in Spruce water, 266 mg

Cl/L in House water, 327 mg Cl/L in Cedar water (Table 5), all of which were markedly lower than the acute LC50s reported above (Table 4). The unreplicated chronic LC50 for *N. triangulifer* in White Clay water was 707 mg Cl/L based on Spearman-Karber and Probit. The chronic LC50 values for *N. triangulifer* did not differ significantly among Spruce, House and Cedar waters, although the ANOVA P value was nearly significant ($0.07 < P < 0.10$)

The acute-to-chronic ratios (ACR) for survivorship (LC50) of *N. triangulifer* were 6.9 for Spruce, 11.8 for House, and 4.2 for Cedar water based on Spearman-Karber, and 4.9 for Spruce, 8.1 for House, and 4.3 for Cedar water based on Probit (Table 5). The ACR for survivorship of *N. triangulifer* was 2.4-2.7 for White Clay water.

Table 5. Chronic (whole life cycle) LC50 estimates (geometric mean) for four mayfly species exposed to various chloride concentrations in four different source waters that differed in hardness (shown in parentheses). Ratios represent acute-to-chronic ratios (ACR) - acute survivorship versus chronic survivorship (acute LC50:chronic LC50). Data for WCC represent only one replicate.

	Spruce (6)		House (94)		Cedar (212)		WCC (89)	
Spearman-Karber	mean	ratio	mean	ratio	mean	ratio	mean	ratio
N. triangulifer	103	6.9	196	11.8	365	4.2	707	2.4
A. semirufum	73	1.0	394	4.6	173	7.8	402	3.1
P. fragile	189	2.4	363	6.2	332	6.5	294	6.4
M. modestum							90	41.3
Probit	mean	ratio	mean	ratio	mean	ratio	mean	ratio
N. triangulifer	143	4.9	266	8.1	327	4.3	707	2.7
A. semirufum	134	0.8	385	4.7	198	6.7	387	2.8
P. fragile	209	2.3	396	5.3	329	5.4	288	5.8
M. modestum							146	23.5

Based on probit analyses, the chronic LC20 for *N. triangulifer* was 109 mg Cl/L in Spruce water, 175 mg Cl/L in House water, and 188 mg Cl/L in Cedar water (Table 6). The unreplicated chronic LC20 for *N. triangulifer* in White Clay water was 686 mg Cl/L based on Probit. The acute-to-chronic ratios (ACR) for survivorship (acute LC50:chronic LC20) of *N. triangulifer* were 6.5 for Spruce, 12.3 for House, and 7.6 for Cedar water (Table 6). The ACR for survivorship of *N. triangulifer* was 2.7 for White Clay water.

Table 6. Chronic (whole life cycle) LC20 estimates (geometric mean) for four mayfly species exposed to various chloride concentrations in four different source waters that differed in hardness (shown in parentheses). Ratios represent acute-to-chronic ratios (ACR) - acute survivorship versus chronic survivorship (acute LC50:chronic LC20). Data for WCC represent only one replicate.

	Spruce (6)		House (94)		Cedar (212)		WCC (89)	
Probit	mean	ratio	mean	ratio	mean	ratio	mean	ratio
N. triangulifer	109	6.5	175	12.3	188	7.6	686	2.7
A. semirufum	114	0.9	279	6.6	128	10.4	224	4.8
P. fragile	168	2.8	332	6.4	245	7.2	217	7.7
M. modestum							138	24.9

Anafroptilum semirufum

The NOEC for survivorship of *A. semirufum* was 125 mg Cl/L in Spruce water, 250 mg Cl/L in House water, and 250 mg Cl/L in Cedar water (Figures 3 and 4). Statistical analyses are not possible for White Clay because there was only one replicate, and the intermediate responses at 250 and 500 mg Cl/L make it impossible to suggest a “non-statistical” NOEC for survivorship of *A. semirufum* in White Clay water (Figure 3). Survivorship dropped in each of the next higher treatments. Thus, the LOEC for survivorship of *A. semirufum* was 250 mg Cl/L in Spruce water (3.5% survivorship), 500 mg Cl/L in House water (29% survivorship), and 500 mg Cl/L in Cedar water (1.5% survivorship). Again, it was impossible to suggest “non-statistical” LOEC for survivorship of *A. semirufum* in White Clay water, but survivorship was 0% at 1000 mg Cl/L (Figure 3).

Based on Spearman-Karber analyses, the chronic LC50 for *A. semirufum* was 73 mg Cl/L in Spruce water, 394 mg Cl/L in House water, and 173 mg Cl/L in Cedar water (Table 5). Based on Probit analyses, the chronic LC50 for *A. semirufum* was 134 mg Cl/L in Spruce water, 385 mg Cl/L in House water, and 198 mg Cl/L in Cedar water. All of these chronic LC50 values were markedly lower that the acute LC50s reported above (Table 4). The unreplicated chronic LC50 for *A. semirufum* in White Clay water was 402 mg Cl/L based on Spearman-Karber, and 387 mg Cl/L based on Probit. The chronic LC50 values for *A. semirufum* differed significantly among Spruce, House and Cedar waters. Both Spearman-Karber and Probit values from Spruce were significantly lower than from House, and Cedar was either different from House (Probit) or comparable to both Spruce and House (Spearman-Karber).

The acute-to-chronic ratios (ACR) for survivorship (LC50:LC50) of *A. semirufum* were 1.0 for Spruce, 4.6 for House, and 7.8 for Cedar water based on Spearman-Karber, and 0.8 for Spruce, 4.7 for House, and 6.7 for Cedar water based on Probit (Table 5). The ACR for survivorship of *A. semirufum* was 2.8-3.1 for White Clay water.

Based on probit analyses, the chronic LC20 for *A. semirufum* was 114 mg Cl/L in Spruce water, 279 mg Cl/L in House water, and 128 mg Cl/L in Cedar water (Table 5). The unreplicated chronic LC20 for *A. semirufum* in White Clay water was 224 mg Cl/L based on Probit. The chronic LC20 value for *A. semirufum* was comparable to the chronic LC20 value estimated for *N. triangulifer* in Spruce water (114 vs 109 mg Cl/L), greater than the chronic LC20 value estimated for *N. triangulifer* in House water (279 vs 175 mg Cl/L) and less than the chronic LC20 value estimated for *N. triangulifer* in Cedar water (128 vs 188 mg Cl/L). The acute-to-chronic ratios (ACR) for survivorship (LC50:LC20) of *A. semirufum* were 0.9 for Spruce, 6.6 for House, and 10.4 for Cedar water (Table 6). The ACR for survivorship of *A. semirufum* was 4.8 for White Clay water.

Procloeon fragile

The NOEC for survivorship of *P. fragile* was 125 mg Cl/L in Spruce water, 250 mg Cl/L in House water, and 250 mg Cl/L in Cedar water (Figures 5 and 6). Statistical analyses are not possible for White Clay because there was only one replicate, but the "non-statistical" NOEC for survivorship of *P. fragile* was 250 mg Cl/L in White Clay water (Figure 5). Survivorship dropped in each of the next higher treatments. Thus, the LOEC for survivorship of *P. fragile* was 250 mg Cl/L in Spruce water (18.5% survivorship), 500 mg Cl/L in House water (15% survivorship), and 500 mg Cl/L in Cedar water (7% survivorship) (Figures 5 and 6). The "non-statistical" LOEC for survivorship of *P. fragile* appears to be 500 mg Cl/L in White Clay water (4% survivorship) (Figure 5).

Based on Spearman-Karber analyses, the chronic LC50 for *P. fragile* was 189 mg Cl/L in Spruce water, 363 mg Cl/L in House water, and 332 mg Cl/L in Cedar water (Table 5). Based on Probit analyses, the chronic LC50 for *P. fragile* was 209 mg Cl/L in Spruce water, 396 mg Cl/L in House water, and 329 mg Cl/L in Cedar water. All of these chronic LC50 values were markedly lower that the acute LC50s reported above (Table 4). The unreplicated chronic LC50 for *P. fragile* in White Clay water was 294 mg Cl/L based on Spearman-Karber, and 288 mg Cl/L based on Probit. The chronic LC50 values for *P. fragile* differed significantly among Spruce, House and Cedar waters. Both Spearman-Karber and Probit values from Spruce were significantly lower than from House, and Cedar was either comparable to both Spruce and House (Probit) or comparable to House but greater than Spruce (Spearman-Karber).

The acute-to-chronic ratios (ACR) for survivorship (LC50:LC50) of *P. fragile* were 2.4 for Spruce, 6.2 for House, and 6.5 for Cedar water based on Spearman-Karber, and 2.3 for Spruce, 5.3 for House, and 5.4 for Cedar water based on Probit (Table 5). The ACR for survivorship of *P. fragile* was 5.8-6.4 for White Clay water.

Based on probit analyses, the chronic LC20 for *P. fragile* was 168 mg Cl/L in Spruce water, 332 mg Cl/L in House water, and 245 mg Cl/L in Cedar water (Table 5). The unreplicated chronic LC20 for *P. fragile* in White Clay water was 217 mg Cl/L based on Probit. The chronic LC20 value for *P. fragile* was greater than the chronic LC20 value estimated for *N. triangulifer* in Spruce water (168 vs 109 mg Cl/L), House water (332 vs 175 mg Cl/L), and Cedar water (245 vs 188 mg Cl/L). The acute-to-chronic ratios (ACR) for survivorship (LC50:LC20) of *P. fragile* were 2.8 for Spruce, 6.4 for House, and 7.2 for Cedar water (Table 6). The ACR for survivorship of *P. fragile* was 7.7 for White Clay water.

Macaffertium modestum

The data for *M. modestum* represents only two replicates (versus 4 for the other species), and only for White Clay water. The NOEC for survivorship of *M. modestum* was 125 mg Cl/L in White Clay water, and the LOEC for survivorship of *M. modestum* was 250 mg Cl/L (Figure 7).

Based on Spearman-Karber analyses, the chronic LC50 for *M. modestum* was 90 mg Cl/L in White Clay water (none of the other waters were tested; Table 5). Based on Probit analyses, chronic LC50 for *M. modestum* was 146 mg Cl/L in White Clay water. Both values are markedly lower that the acute LC50s reported in Table 4 (i.e., 3718 and 3433 mg Cl/L, respectively). The acute-to-chronic ratios (ACR) for survivorship (acute LC50:chronic LC50) of *M. modestum* in White Clay water was 41.3 based on Spearman-Karber, and 23.5 based on Probit (Table 5).

Based on probit analyses, the chronic LC20 for *M. modestum* was 138 mg Cl/L in White Clay water. The chronic LC20 value for *M. modestum* was less than the chronic LC20 value estimated for all three baetid mayflies in White Clay water, but these White Clay tests were not well replicated and differences should be interpreted with caution. The acute-to-chronic ratio (ACR) for survivorship (acute LC50:chronic LC20) of *M. modestum* was 24.9 for White Clay water.

Development time (median days from 1st instar to adult emergence)

Neocloeon triangulifer

The no-observed-effect concentration (NOEC) for development time of *N. triangulifer* was 125 mg Cl/L in Spruce water, 250 mg Cl/L in House water, and 125 mg Cl/L in Cedar water (Figures 8, 9, 10). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for development time of *N. triangulifer* appears to be 250 mg Cl/L in White Clay water (Figure 11). Development time increased in each of the next higher treatments. Thus, the lowest-observed-effect concentration (LOEC) for development time of *N. triangulifer* was 250 mg Cl/L in Spruce water, 500 mg Cl/L in House water, and 250 mg Cl/L in Cedar water (Figures 8, 9, 10). The "non-statistical" LOEC for development time of *N. triangulifer* was 500 mg Cl/L in White Clay water (Figure 11). The highest concentration of chloride with survivors (i.e., 250 or 500 mg

Cl/L) increased development time 9.8-11.4 d compared to the control. For example, development time increased from 33.5 d for the control to 43.3 d in Spruce water and from 29.9 d for the control to 41.3 d in Cedar water.

We used simple linear regressions to estimate the EC20 for development time (i.e., the Cl concentration needed to increase development time for *N. triangulifer* by 20%). The EC20 for development time was 191 mg Cl/L in Spruce water, 429 mg Cl/L in House water, and 323 mg Cl/L in Cedar water (Table 7). The unreplicated chronic EC20 for *N. triangulifer* in White Clay water was 492 mg Cl/L. The EC20s for development time for *N. triangulifer* differed significantly among Spruce, House and Cedar waters, with values from Spruce significantly less than from House, while Cedar was comparable to both Spruce and House.

EC20s for development time for *N. triangulifer* were 72-145% greater than the LC20s described for Spruce, House, and Cedar water (Tables 6 and 7). The EC20 for development time for *N. triangulifer* in White Clay water was 28% less than the LC20 (Tables 6 and 7), but this was unreplicated and interpreted with caution. The acute-to-chronic ratios (ACR) for development time (acute LC50:chronic LC20) of *N. triangulifer* were 3.7 for Spruce, 5.0 for House, and 4.4 for Cedar water (Table 7). The ACR for development time of *N. triangulifer* was 3.8 for White Clay water.

Table 7. Chronic (whole life cycle) EC20 estimates (geometric mean) for larval development time for three mayfly species exposed to various chloride concentrations in four different source waters that differed in hardness (shown in parentheses). Ratios represent acute-to-chronic ratios (ACR) - acute survivorship versus chronic development time (acute LC50:chronic EC20). Data for WCC represent only one replicate.

	Spruce (6)		House (94)		Cedar (212)		WCC (89)	
Probit	mean	ratio	mean	ratio	mean	ratio	mean	ratio
N. triangulifer	191	3.7	429	5.0	323	4.4	492	3.8
A. semirufum – females	369	0.3	423	4.3	255	5.2	542	2.0
A. semirufum – males	299	0.4	447	4.1	331	4.0	842	1.3
P. fragile – females	291	1.6	402	5.2	340	5.2	541	3.1
P. fragile– males	229	2.1	445	4.7	344	5.1	637	2.6

Anafroptilum semirufum

Development time for *A. semirufum* appeared to increase with increasing chloride in all four source waters, but the interpretation of these trends was complicated by no statistically significant differences among treatments for males and females in Spruce, and males in Cedar. The NOEC for development time of *A. semirufum* was 250 mg Cl/L in Spruce water, House water, and Cedar water (Figures 12, 13, 14). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for development time of *A. semirufum* appears to be 250 or 500 mg Cl/L in White Clay water (Figure 15). The LOEC for development time of *A. semirufum* could not be estimated for Spruce water because no adults emerged at 500 mg Cl/L, and development time at 250 mg Cl/L did not differ significantly from the control (survivorship was nearly 0% at 250 mg Cl/L in Spruce water) (Figure 12). The LOEC for development time of *A. semirufum* was 500 mg Cl/L in House water and 500 mg Cl/L in Cedar water (Figures 13, 14). The "non-statistical" LOEC for survivorship of *A. semirufum* was 500 or 1000 mg Cl/L in White Clay water (Figure 15). The highest concentration of chloride with survivors (i.e., 250, 500, or 1000 mg Cl/L) increased development time 2-12 d compared to the control. For example, development time of *A. semirufum* increased from 22-23 d for the control to 28 d for males and 33 d for females in House water. Similarly development time of *A. semirufum* increased from 22-23 d for the control to 29 d for males and 31 d for females in Cedar water.

The EC20 for development time for females of *A. semirufum* was 369 in Spruce water, 423 in House water, and 255 in Cedar water. The EC20 for development time for males of *A. semirufum* was 299 in Spruce water, 447 in House water, and 331 in Cedar water. The unreplicated chronic EC20 for *A. semirufum* in White Clay water was 542 mg Cl/L for females, and 842 mg Cl/L for males. The EC20s for development time for *A. semirufum* did not differ significantly among Spruce, House and Cedar waters.

The EC20s for development time for *A. semirufum* were 162-224% greater than LC20s described for *A. semirufum* in Spruce, 52-60% greater than LC20s for House, and 99-159% greater than LC20s for Cedar water (Tables 6 and 7). These chronic EC20 values for development time of *A. semirufum* females were greater than the chronic EC20 values estimated for *N. triangulifer* in Spruce water (369 vs 191 mg Cl/L), and comparable to *N. triangulifer* in House water (423 vs 429 mg Cl/L) and Cedar water (255 vs 323 mg Cl/L). The acute-to-chronic ratios (ACR) for development time (acute LC50:chronic LC20) of *A. semirufum* were 0.3-0.4 for females and males in Spruce, 4.1-4.3 for females and males in House, and 5.2 for females and 4.0 for males in Cedar (Table 7). The ACR for development time of *A. semirufum* was 2.0 for females and 1.3 for males in White Clay water.

Procloeon fragile

The NOEC for development time of *P. fragile* was 125 mg Cl/L in Spruce water, 250 mg Cl/L in House water, and 125 mg Cl/L for males and 250 for females in Cedar water (Figures 16, 17, 18). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for development time of *P. fragile* appears to be 250 mg Cl/L in White Clay water (Figure 19). Development time increased in each of the next higher treatments. Thus, the LOEC for development time of *P. fragile* was 250 mg Cl/L in Spruce water, 500 mg Cl/L in House water, and 250 mg Cl/L for males and 500 for females in Cedar water (Figures 16, 17, 18). The "non-statistical" LOEC for development time of *P. fragile* was 500 mg Cl/L in White Clay water, although no females survived at that concentration (Figure 19). The highest concentration of chloride with survivors (i.e., 250 or 500 mg Cl/L) increased development time 6-12 d compared to the control. For example, development time of *P. fragile* increased from ≈25 d for the control to 31 d for males and 37 d for females in Spruce water. Similarly development time increased from ≈26 d for the control to 35 d for males and 37 d for females in Cedar water.

The EC20 for development time for females of *P. fragile* was 291 in Spruce water, 402 in House water, and 340 in Cedar water. The EC20 for development time for males of *P. fragile* was 229 in Spruce water, 445 in House water, and 344 in Cedar water. The unreplicated chronic EC20 for *P. fragile* in White Clay water was 541 mg Cl/L for females, and 637 mg Cl/L for males. The EC20s for development time for females of *P. fragile* did not differ significantly among Spruce, House and Cedar waters whereas the EC20 for development time for males of *P. fragile* from Spruce water was significantly less than from House, while Cedar was comparable to both Spruce and House.

The EC20s for development time for *P. fragile* were 36-73% greater than LC20s described for *P. fragile* in Spruce, 21-34% greater than LC20s for House, and 39-40% greater than LC20s for Cedar water (Tables 6 and 7). These chronic EC20 values for development time of *P. fragile* females were greater than the chronic EC20 values estimated for *N. triangulifer* in Spruce water (291 vs 191 mg Cl/L), and comparable to *N. triangulifer* in House water (402 vs 429 mg Cl/L) and Cedar water (340 vs 323 mg Cl/L). The acute-to-chronic ratios (ACR) for development time (acute LC50:chronic LC20) of *P. fragile* were 1.6 for females and 2.1 for males in Spruce, 5.2 for females and 4.7 for males in House, and 5.2 for females and 5.1 for males in Cedar (Table 7). The ACR for development time of *P. fragile* was 3.1 for females and 2.6 for males in White Clay water.

Adult body size (mg dry mass)

We generally observed either no difference or an increase in adult body size as chloride concentration increased, across *N. triangulifer*, *A. semirufum*, and *P. fragile* and the four source waters (Spruce, House, Cedar, and White Clay; Figues 8 - 19). The only exception was males of *A. semirufum* in House water, where body size decreased. We interpreted the increases in body size not as adverse effects but as a response of very few individuals surviving in higher concentrations of chloride. It appears that increases in development time generally compensate for the slower growth rate, resulting in no change or an increase in adult body size as chloride concentration increased. As a result, we did not identify a NOEC or LOEC for adult body size reacting negatively to exposure to chloride.

Instantaneous growth rate (d^{-1})

Neocloeon triangulifer

The no-observed-effect concentration (NOEC) for Instantaneous Growth Rate (IGR) of *N. triangulifer* was 125 mg Cl/L in Spruce and Cedar water, and 250 mg Cl/L in House water (Figures 8, 9, 10). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for IGR of *N. triangulifer* appears to be 125 or 250 mg Cl/L in White Clay water (Figure 11). IGR increased in each of the next higher treatments. Thus, the lowest-observed-effect concentration (LOEC) for IGR of *N. triangulifer* was 250 mg Cl/L in Spruce and Cedar water, and 250 mg Cl/L in House water (Figures 8, 9, 10). The "non-statistical" LOEC for IGR of *N. triangulifer* was 250 or 500 mg Cl/L in White Clay water (Figure 11).

IGR for *N. triangulifer* decreased on average 23% between the control and the highest chloride concentration that could be examined (250 or 500 mg Cl/L). IGR averaged 0.23-0.25 d^{-1} in control water and 0.18-0.19 d^{-1} in the highest chloride water with emerging adults. The EC20 for IGR of *N. triangulifer* was 299 mg Cl/L in Spruce water, 564 mg Cl/L in House water, 442 mg Cl/L in Cedar water (Table 8). The unreplicated chronic EC20 for *N. triangulifer* in White Clay water was 590 mg Cl/L. The EC20s for IGR for *N. triangulifer* differed significantly among Spruce, House and Cedar waters, with values from Spruce significantly less than from House, while Cedar was comparable to both Spruce and House.

Table 8. Chronic (whole life cycle) EC20 estimates (geometric mean) for Instantaneous Growth Rate (IGR) for three mayfly species exposed to various chloride concentrations in four different source waters that differed in hardness (shown in parentheses). Ratios represent acute-to-chronic ratios (ACR) - acute survivorship versus chronic IGR (acute LC50:chronic EC20). Data for WCC represent only one replicate.

	Spruce (6)		House (94)		Cedar (212)		WCC (89)	
Probit	mean	ratio	mean	ratio	mean	ratio	mean	ratio
N. triangulifer	299	2.4	564	3.8	442	3.2	590	3.2
A. semirufum – females	494	0.2	538	3.4	386	3.5	693	1.6
A. semirufum – males	424	0.3	566	3.2	457	2.9	1030	1.0
P. fragile – females	435	1.1	576	3.7	444	4.0	716	2.3
P. fragile– males	340	1.4	594	3.6	459	3.9	859	1.9

EC20s for IGR for *N. triangulifer* were 174% greater than LC20s described for *N. triangulifer* in Spruce, 222% greater than LC20s for House, and 135% greater than LC20s for Cedar water (Tables 6 and 8). The acute-to-chronic ratios (ACR) for IGR (acute LC50:chronic LC20) of *N. triangulifer* was 2.4 in Spruce, 3.8 in House, and 3.2 in Cedar (Table 8). The ACR for IGR of *N. triangulifer* was 3.2 in White Clay water.

Anafroptilum semirufum

Instantaneous Growth Rate for *A. semirufum* appeared to decrease with increasing chloride in all four source waters, but the interpretation of these trends was complicated, as with development time, by no significant differences (males and females in Spruce, males in Cedar). The NOEC for IGR of *A. semirufum* was 250 mg Cl/L in Spruce water, House water, and Cedar water (Figures 12, 13, 14). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for IGR of *A. semirufum* appears to be 250 or 500 mg Cl/L in White Clay water (Figure 15). The LOEC for IGR of *A. semirufum* could not be estimated for Spruce water because no adults emerged at 500 mg Cl/L, and IRG at 250 mg Cl/L did not differ significantly from the control (survivorship was nearly 0% at 250 mg Cl/L in Spruce water). The LOEC for IGR of *A. semirufum* was 500 mg Cl/L in House water and 500 mg Cl/L in Cedar water (Figures 12, 13, 14). The "non-statistical" LOEC for survivorship of *A. semirufum* was 500 or 1000 mg Cl/L in White Clay water (Figure 15).

IGR for *A. semirufum* decreased on average 21% between the control and the highest chloride concentration that could be examined (250, 500 or 1000 mg Cl/L). IGR averaged 0.30-0.33 d^{-1} in control water and 0.19-0.29 d^{-1} in the highest chloride water with emerging adults. The EC20 for IGR for females of *A. semirufum* was 494 in Spruce water, 538 in House water, and 386 in Cedar water. The EC20 for IGR for males of *A. semirufum* was 424 in Spruce water, 566 in House water, and 457 in Cedar water. The unreplicated chronic EC20 for *A. semirufum* in White Clay water was 693 mg Cl/L for females, and 1030 mg Cl/L for males. The EC20s for IGR for *A. semirufum* did not differ significantly among Spruce, House and Cedar waters.

The EC20s for IGR for *A. semirufum* were 272-333% greater than LC20s described for Spruce, 93-103% greater than LC20s for House, and 202-257% greater than LC20s for Cedar (Tables 6 and 7). These chronic EC20 values for IGR of *A. semirufum* females were greater than the chronic EC20 values estimated for *N. triangulifer* in Spruce water (494 vs 299 mg Cl/L), and comparable to *N. triangulifer* in House water (538 vs 564 mg Cl/L) and Cedar water (386 vs 442 mg Cl/L). The acute-to-chronic ratios (ACR) for IGR (acute LC50:chronic LC20) of *A. semirufum* were 0.2-0.3 for females and males in Spruce, 3.2-3.4 for females and males in House, and 3.5 for females and 2.9 for males in Cedar (Table 8). The ACR for IGR of *A. semirufum* was 1.6 for females and 1.0 for males in White Clay water.

Procloeon fragile

The NOEC for IGR of *P. fragile* was 125 mg Cl/L in Spruce water, 250 mg Cl/L in House water, and 125 mg Cl/L for males and 250 for females in Cedar water (Figures 16, 17, 18). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for IGR of *P. fragile* appears to be 250 mg Cl/L in White Clay water (Figure 19). IGR decreased in each of the next higher treatments. Thus, the LOEC for IGR of *P. fragile* was 250 mg Cl/L in Spruce water, 500 mg Cl/L in House water, and 250 mg Cl/L for males and 500 for females in Cedar water (Figures 16, 17, 18). The "non-statistical" LOEC for IGR of *P. fragile* was 500 mg Cl/L in White Clay water, although no females survived at that concentration (Figure 19).

IGR for *P. fragile* decreased on average 23% between the control and the highest chloride concentration that could be examined (250, 500 or 1000 mg Cl/L). IGR averaged 0.27-0.29 d^{-1} in control water and 0.20-0.23 d^{-1} in the highest chloride water with emerging adults. The EC20 for IGR for females of *P. fragile* was 435 in Spruce water, 576 in House water, and 444 in Cedar water. The EC20 for IGR for males of *P. fragile* was 340 in Spruce water, 594 in House water, and 459 in Cedar water. The unreplicated chronic EC20 for *P. fragile* in White Clay water was 716 mg Cl/L for females, and 859 mg Cl/L for males. The EC20s for IGR for females of *P. fragile* did not differ significantly among Spruce, House and Cedar waters whereas the EC20s for IGR for males of *P. fragile* from Spruce water were significantly less than from House, while Cedar was comparable to both Spruce and House.

The EC20s for IGR for *P. fragile* were 102-159% greater than LC20s described for Spruce, 73-79% greater than LC20s for House, and 81-87% greater than LC20s for Cedar (Tables 6 and 7). These chronic EC20 values for IGR of *P. fragile* females were greater than the chronic EC20 values estimated for *N. triangulifer* in Spruce water (435 vs 299 mg Cl/L), and comparable to *N. triangulifer* in House water (576 vs 564 mg Cl/L) and Cedar water (444 vs 442 mg Cl/L). The acute-to-chronic ratios (ACR) for IGR (acute LC50:chronic LC20) of *P. fragile* were 1.1 for females and 1.4 for males in Spruce, 3.7 for females and 3.6 for males in House, and 4.0 for females and 3.9 for males in Cedar (Table 8). The ACR for IGR of *P. fragile* was 2.3 for females and 1.9 for males in White Clay water.

Population Growth Rate (eggs individual^{-1} d^{-1})

The differences among chloride treatments for PGR were very similar to those observed for survivorship (Figures 1-7 versus 8-19). It is important to note that PGR is zero when chronic survivorship is zero, so there are no data illustrated at chloride concentrations > 1000 mg Cl/L We used Probit analyses similar to those for survivorship to estimate the EC20 (i.e., the Cl concentration needed to reduce PGR by 20%).

Neocloeon triangulifer

The NOEC for PGR of *N. triangulifer* was 250 mg Cl/L in Spruce water, 500 mg Cl/L in House water, and 250 mg Cl/L in Cedar water (Figures 8, 9, 10). Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for PGR of *N. triangulifer* appears to be 500 mg Cl/L in White Clay water, which translates to an average of 25.7 eggs individual^{-1} d^{-1} (Figure 11). The LOEC for PGR of *N. triangulifer* could not be estimated for Spruce, House, or White Clay water because no larvae survived or adults emerged at the chloride concentration above the NOEC. There were negative trends at higher chloride concentrations for Spruce and House, but none of the differences between concentrations were statistically significant. The LOEC was 500 mg Cl/L for *N. triangulifer* in Cedar waters, which averaged 10.5 eggs individual^{-1} d^{-1} or a decrease 66% from the NOEC (31 eggs individual^{-1} d^{-1}).

The EC20 for PGR of *N. triangulifer* was 107 mg Cl/L in Spruce water, 176 mg Cl/L in House water, 190 mg Cl/L in Cedar water (Table 9). A chronic EC20 for *N. triangulifer* in White Clay water could not be estimated. The EC20s for PGR of *N. triangulifer* did not differ significantly among Spruce, House and Cedar waters. The EC20s for PGR of *N. triangulifer* were similar to LC20s of *N. triangulifer* in Spruce (107 vs 109 mg Cl/L), House (176 vs 175 mg Cl/L), and Cedar (190 vs 188 mg Cl/L) (Tables 6, 9). The acute-to-chronic ratios (ACR) for PGR (acute LC50:chronic EC20) of *N. triangulifer* were 6.6 for Spruce, 12.2 for House, and 7.5 for Cedar water (Table 9).

Table 9. Chronic (whole life cycle) EC20 estimates (geometric mean) for Population Growth Rate (PGR) for three mayfly species exposed to various chloride concentrations in four different source waters that differed in hardness (shown in parentheses). Ratios represent acute-to-chronic ratios (ACR) - acute survivorship versus chronic PRG (acute LC50:chronic EC20). Data for WCC represent only one replicate.

	Spruce (6)		House (94)		Cedar (212)		WCC (89)	
Probit	mean	ratio	mean	ratio	mean	ratio	mean	ratio
N. triangulifer	107	6.6	176	12.2	190	7.5	-	-
A. semirufum	62	1.7	298	6.1	135	9.9	202	5.3
P. fragile	177	2.7	363	5.8	199	8.9	241	6.9

Anafroptilum semirufum

The NOEC for PGR of *A. semirufum* was 250 mg Cl/L in Spruce, House, and Cedar water. Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for PGR of

A. semirufum appears to be 125 mg Cl/L in White Clay water, which translates to an average of 23 eggs individual^{-1} d^{-1} (Figure 15). The LOEC for PGR of *A. semirufum* could not be estimated for Spruce water because no larvae survived or adults emerged at the chloride concentration above the NOEC. The LOEC for PGR of *A. semirufum* was 500 mg Cl/L in House and Cedar water, which averaged 1-8 eggs individual^{-1} d^{-1} or a decrease of 67-96% from the NOEC (21-23 eggs individual^{-1} d^{-1}). Statistical analyses are not possible for White Clay, but the "non-statistical" LOEC for PGR of *A. semirufum* appears to be 250 mg Cl/L in White Clay water.

The EC20 for PGR of *A. semirufum* was 62 mg Cl/L in Spruce water, 298 mg Cl/L in House water, 135 mg Cl/L in Cedar water (Table 9). The unreplicated chronic EC20 for *A. semirufum* in White Clay water was 202 mg Cl/L based on Probit. The EC20s for PGR for *A. semirufum* did not differ significantly among Spruce, House and Cedar waters.

The EC20s for PGR of *A. semirufum* were less than the LC20s of *A. semirufum* in Spruce (62 vs 114 mg Cl/L), but relatively similar in House (298 vs 279 mg Cl/L) and Cedar (135 vs 128 mg Cl/L) (Tables 6, 9). The chronic EC20 for PGR of *A. semirufum* was less than the chronic EC20 value estimated for *N. triangulifer* in Spruce water (62 vs 107 mg Cl/L) and in Cedar water (135 vs 190 mg Cl/L), and greater than the chronic EC20 value estimated for *N. triangulifer* in House water (298 vs 176 mg Cl/L). The acute-to-chronic ratios (ACR) for PGR (acute LC50:chronic EC20) of *A. semirufum* were 1.7 for Spruce, 6.1 for House, and 9.9 for Cedar water (Table 9). The ACR for PGR of *A. semirufum* was 5.3 for White Clay water (Table 9).

Procloeon fragile

The NOEC for PGR of *P. fragile* was 125 mg Cl/L in Spruce water, and 250 mg Cl/L in House and Cedar water (Figures 16, 17, 18). This translates to an average of 17-18 eggs individual^{-1} d^{-1}. Statistical analyses are not possible for White Clay, but the "non-statistical" NOEC for PGR of *P. fragile* appears to be 250 mg Cl/L in White Clay water (Figure 19). PGR decreased in each of the next higher treatments. Thus, the LOEC for PGR of *P. fragile* was 250 mg Cl/L in Spruce water, and 500 mg Cl/L in House and Cedar water (Figures 16, 17, 18). PGR of *P. fragile* at the LOEC averaged 2-5 eggs individual^{-1} d^{-1} or a decrease of 71-89% from the NOEC (17-18 eggs individual^{-1} d^{-1}). The "non-statistical" LOEC for PGR of *P. fragile* could not be estimated for White Clay water because no larvae survived or adults emerged at the chloride concentration above the NOEC (Figure 19).

The chronic EC20 for PGR of *P. fragile* was 177 mg Cl/L in Spruce water, 363 mg Cl/L in House water, and 199 mg Cl/L in Cedar water (Table 9). The unreplicated chronic EC20 for *P. fragile* in White Clay water was 241 mg Cl/L based on Probit. The EC20s for PGR of *P. fragile* did not differ significantly among Spruce, House and Cedar waters.

The EC20s for PGR of *P. fragile* were comparable to the LC20s of *P. fragile* in Spruce (177 vs 168 mg Cl/L), House (363 vs 332 mg Cl/L), and Cedar (199 vs 245 mg Cl/L) (Tables 6, 9). The chronic EC20 value for *P. fragile* was greater than the chronic EC20 value estimated for *N. triangulifer* in Spruce water (177 vs 107 mg Cl/L), House water (363 vs 176 mg Cl/L), and comparable to the chronic EC20 value estimated for *N. triangulifer* in Cedar water (199 vs 190 mg Cl/L). The acute-to-chronic ratios (ACR) for PGR (acute LC50: chronic EC20) of *P. fragile* were 2.7 for Spruce, 5.8 for House, and 8.9 for Cedar water (Table 9). The ACR for PGR of *P. fragile* was 6.9 for White Clay water.

Summary of Nonlethal Responses to Chloride

Both lethal and nonlethal responses to chloride were measured during the chronic, lifecycle experiments. Nonlethal responses measured were development time (d), adult body size (mg), Instantaneous Growth Rate (IGR, d^{-1}), and Population Growth Rate (PGR). Development time, IGR, and PGR all exhibited what would be interpreted as a negative response to elevated chloride – development time from 1st instar to adult increased, while IGR and PGR decreased. Responses for development time and IGR were relatively linear (Figure 20) while PGR was more sigmoidal like survivorship. In general, EC20s for development time and IGR were greater than LC20, making them less sensitive measures of mayfly responses to chloride (i.e., it took less chloride to decrease survivorship by 20% than to increase development time and decrease IGR by 20%. Survivorship and PGR were relatively similar in most cases, presumably because survivorship appears to be more dynamic relative to development time and body size, and therefore dominates the calculation of PGR in chloride tests. In a few cases, the EC20 for PGR was less than the LC20 (i.e., *A. semirufum* in Spruce and *P. fragile* in Cedar), suggesting that development time and/or body size may have contributed more to PGR in some other tests. However, given the available data, it appears that survivorship is the most sensitive measure of chronic responses to elevated chloride for these three species.

Discussion

Mayfly responses to acute exposures to chloride

Our acute experiments involving six different mayfly species (representing six genera in four families) resulted in two general observations. First, three of six mayfly species (i.e., the baetid mayflies *N. triangulifer*, *A. semirufum*, *P. fragile*) are relatively sensitive to chloride in a brief (48 h) exposure compared with the other three species (i.e., *E. invaria*, *L. cupida*, *M. modestum*). Second, acute chloride toxicity for five of six mayflies examined (*N. triangulifer*, *A. semirufum*, *P. fragile*, *E. invaria*, *M. modestum*, but not *L. cupida*) is greater when the test waters are soft relative to moderately hard and/or hard waters. There may be differences between moderately hard and hard waters, but these differences were not statistically significant given the variance observed among tests and limited number of tests per water. There was general agreement between EPA tolerance values (TV, Table 2) and acute chloride sensitivity, with baetids being more sensitive than the other families. However, this relationship is not apparent when using PA tolerance values (Table 2), due at least in part to the high PA TV assigned *Procloeon fragile*, and the low PA TV assigned to *Ephemerella invaria*. (Note: the TV assigned to *Procloeon fragile* and *Ephemerella invaria* were not species specific values, but were based on a genus-level value representing all of the Pennsylvania species in the each genus.)

The only mayfly species examined in our study that has also been included in other acute chloride toxicity studies is *N. triangulifer*. Our results for *N. triangulifer* in House (2141-2301 mg Cl/L) and White Clay water (1667-1885 mg Cl/L, Table 4) were comparable to the 48-h LC50 of 1869-2155 mg Cl/L we observed in an earlier study of 1st instar *N. triangulifer* in White Clay water (SWRC unpublished data). LC50s for Cedar (1420-1551 mg Cl/L) were lower than we observed in an earlier study of 1st instar *N. triangulifer* in White Clay water (SWRC unpublished data). Our results for *N. triangulifer* in House, Cedar, and White Clay were greater, in some cases double, the 96-h LC50 of 1062 Cl/L reported by Soucek and Dickinson (in Duluth 100 water, hardness of 100 mg/L as $CaCO_3$, In Press). We presume (based on our experience) the longer (48-h versus 96-h) exposure time and the warmer (20 versus 25°C) water temperature contribute to the lower LC50 in Soucek and Dickinson (In Press). Our 48-h LC50 values for *N. triangulifer* in House, Cedar, or White Clay water are markedly higher than was recently reported for 1st instar *N. triangulifer* by Struwing et al. (2014; 400 mg Cl/L in moderately hard reconstituted water (MHRW) with a hardness of 80-100 mg/L); however, we do not presently know how to use the Struwing et al. data as their reported chronic values are higher than their acute values. Our 48-h LC50 of 704-712 mg Cl/L in Spruce water (hardness of 8 mg/L as $CaCO_3$) is lower than the 48-h LC50 of 1037-1038 mg Cl/L we observed for 1st instar *N. triangulifer* in Dyberry Creek water (a soft-water stream with a hardness of 22 mg/L as $CaCO_3$) (SWRC unpublished data). Thus, the results from Spruce and Dyberry waters indicate that there was increased sensitivity to Cl when hardness is low relative to moderately hard and hard waters. We observed some difference between our moderately hard (House, White Clay) and hardest water (Cedar), but these differences were not significant. Mount et al. (1997) found that elevated carbonate concentrations (which accompany increased hardness in these study waters) can have toxic effects on some aquatic invertebrates, but the interaction of increased hardness (and carbonate) with chloride was not consistent among our tests. For better comparability across both acute and chronic tests, we will be increasing the duration of future acute toxicity tests involving 1st instar mayflies to 96-h tests.

While all but one of these mayfly species have not been studied previously, acute toxicity of chloride has been examined for a number of aquatic vertebrate (fish and amphibian) and invertebrate (insect and non-insect) species. To better understand the sensitivity of the mayflies examined in our study, we plotted (Figure 21) the acute data for these six mayfly species (i.e., *N. triangulifer*, *A. semirufum*, *P. fragile*, *E. invaria*, *L. cupida*, *M. modestum*) with the data for 53 species included in Table 3 of CCME (2011). For mayflies in moderately hard water, the ranking of acute LC50s for mayflies ranged from about approximately 35 - 80% (Figure 21). Thus, in moderately hard water, these six mayfly species are clearly not the most sensitive aquatic species when exposed to elevated chloride. This result should be interpreted with caution as our tests were only for 48 h whereas many of the other data points represent 96 h exposures (CCME 2011). When these same mayfly species were exposed to chloride in soft water, the ranking for acute LC50s ranged from about <10% to 78%. All species except *L. cupida* were markedly lower in soft (Spruce) water than in one or more of the harder waters, and were among the lowest acute LC50s known for aquatic organisms (Figure 21). If the tests were run for 96 h (versus 48 h as we did in this study), we believe the acute LC50s would decrease by about a factor of 1.5-2.0 (e.g., 2000 to 1000 mg/L), it is possible some of the mayflies (i.e., baetid species) we examined in soft water would then be among the most sensitive aquatic species examined to date.

Mayfly responses to chronic exposures to chloride

Our chronic (whole lifecycle) experiments involving three different mayfly species (representing a single mayfly family, Baetidae) resulted in four general observations. First, among the chronic response variables examined (i.e., survivorship, development time, adult body size, instantaneous growth rate, and population growth rate), survivorship (i.e., LC20) was consistently the most sensitive response variable for chloride (population growth rate was a close second, and it primarily reflects survivorship in chloride exposures). The other response variables showed consistent changes as chloride concentration increased (Figure 20), but a 20% change in these variables appears associated with a >20% increase in mortality. Second, comparing the acute and chronic LC50s, the sensitivity of the three mayfly species appears greater with a longer (48 h versus 22-43 d) exposure to chloride. Third, chronic chloride toxicity for the mayflies examined is greater when the test waters are soft relative to moderately hard and/or hard waters (LC50 differences between waters was not quite significant for *N. triangulifer*, p=0.08-0.09). There may be differences between moderately hard and hard waters, but these differences were generally not statistically significant (except Probit data for *A. semirufum*) given the variance observed among tests and limited number of tests per water. Finally, we were able to complete a partial chronic test (only 50 d, not a full life cycle) for a fourth mayfly, *M. modestum* in White Clay water. Survivorship after 50 d suggests that *M. modestum* is also relatively sensitive to chloride, similar to the three baetid species and in contrast to the acute results for *M. modestum*, which was moderately tolerant (Table 4, Figure 21). This leads us to believe that the relatively consistent acute-to-chronic relationships we observed among the three baetid mayflies may not be applicable to *M. modestum*, and it suggests that acute-to-chronic relationships based on baetid mayflies may not be applicable to other mayflies. Stated another way, for mayflies, moderate tolerance to an acute exposure to chloride may not translate into a moderate tolerance to a chronic exposure to chloride.

As with the acute exposures, the only mayfly species examined in our study that has also been included in other chronic chloride toxicity studies is *N. triangulifer*. Our chronic LC50 results for *N. triangulifer* in House (196-266 mg Cl/L) and Cedar water (327-365 mg Cl/L, Table 5) were more comparable than to the chronic LC50 of 468-582 mg Cl/L we observed in an earlier study of *N. triangulifer* in White Clay water (SWRC unpublished data). Our LC50 value for WCC (707 mg Cl/L) are somewhat higher than the previous chronic LC50 value (468-582 mg Cl/L) we observed for *N. triangulifer* in White Clay water, but this is an unreplicated observation and should be interpreted with caution. Our chronic LC20 results for *N. triangulifer* in House (175 mg Cl/L) and Cedar water (188 mg Cl/L, Table 6) were lower than the chronic LC20 of 315 mg Cl/L we observed in an earlier study of *N. triangulifer* in White Clay water (SWRC unpublished data). Our LC20 results for survivorship of *N. triangulifer* in House (175 mg Cl/L) and Cedar (188 mg Cl/L) water were comparable to the EC20 for %PEN WCF (% surviving to pre-emergent nymph stage when controls finished) of 165 mg Cl/L in Duluth 100 water (hardness of 100 mg/L as $CaCO_3$) reported by Soucek and Dickinson (In Press). Our LC20 results for survivorship of *N. triangulifer* in House and Cedar water are lower than the EC25 recently reported for *N. triangulifer* by Struwing et al. (2014; 399 or 400 mg Cl/L in moderately hard reconstituted water (MHRW) with a hardness of 80-100 mg/L). Again, we do not presently know how to use these data as their reported chronic values are higher than their acute values. Diamond et al (1992) also examined *M. modestum*, but it is not easily compared to our study because the Diamond et al. experiment was at 12 °C (versus 20°C), lasted only 14-d exposures (versus 50 d), and began with larvae with 4-9 mm long bodies (versus 1st instar at <0.5 mm).

Our chronic LC50 for survivorship of *N. triangulifer* in Spruce water (hardness of 8 mg/L as $CaCO_3$) is 103-143 mg Cl/L, which is lower than the LC50 of 362-379 mg Cl/L we observed previously for *N. triangulifer* in Dyberry Creek water (a soft-water stream with a hardness of 22 mg/L as $CaCO_3$) (SWRC unpublished data). Our LC20 for survivorship of *N. triangulifer* in Spruce water (hardness of 8 mg/L as $CaCO_3$) is 109 mg Cl/L, which is lower than the LC20 of 310 mg Cl/L we observed previously for *N. triangulifer* in Dyberry Creek water (SWRC unpublished data). Thus, similar to the acute results, the chronic results from Spruce versus House, Cedar, and WCC, Dyberry versus WCC waters indicate that there was increased sensitivity to a chronic exposure to Cl when hardness is low relative to moderately hard and hard waters. The differences between Spruce and Dyberry suggest that there are also increased sensitivity to a chronic exposure to Cl when hardness is very low relative to moderately soft waters (i.e., Spruce versus Dyberry). We observed some difference between our moderately hard (House) and hardest water (Cedar), but these differences were not significant.

While all but one of these mayfly species had not been studied previously, chronic toxicity of chloride has been examined for a number of aquatic vertebrate (fish and amphibian) and invertebrate (insect and non-insect) species as well as some aquatic algae and plant species. To better understand the sensitivity of the mayflies examined in our study, we plotted the survivorship (LC20) data for these four mayfly species (i.e., *N. triangulifer*, *A. semirufum*, *P. fragile*, *M. modestum*) with the data for 28 species included in Table 5 of CCME (2011). For mayflies in moderately hard water, the ranking for chronic LC20s ranged from <10% to 20% (Figure 22). Thus, in moderately hard water, these four mayfly species are sensitive to exposure to elevated chloride, but are not the most sensitive aquatic species. When the three baetid species were exposed to chloride in soft water, the ranking for chronic LC20s ranged from about 5% to 20%, and were among the lowest chronic values known (Figure 22). Many of the values in CCME (2011) were EC10s such as are shown for three mayflies in Table 10, and incorporating those lower values into Figure 22 would move the mayflies lower into the graph (i.e., making them even more sensitive among the aquatic species examined).

Acute and chronic mayfly responses relative to selected water quality standards

Regulatory criteria have been recommended or enacted based on a number of studies that have used available data (Table 10). Acute criteria range from 405 to 1010 mg/L, and much of this range reflects the Iowa standard model, which is based only on data for *Ceriodaphnia dubia*, and incorporates hardness and sulfate into the derivation of the standard. The Iowa model defined an acute criteria range of 382 – 703 mg/L for PA waters included in our study (Table 10).

Chronic limits range from 120 to 624 mg/L (Table 10), and similar to the acute criteria, much of the range reflects the Iowa standard model for chronic limits. The Iowa model defined a chronic range of 236 – 435 mg/L for waters included in our study (Table 10).

The importance of the toxicity results reported here is that they are from experiments that began with 1st instar mayflies. Thus, the data represent the more sensitive life stages (e.g., Gauss et al. 1985, Williams et al. 1986, Kiffney and Clements 1996, Clark and Clements 2006, Clements et al. 2013) for an important portion of the stream macroinvertebrate fauna commonly used in stream assessments. To date none of the recommended or enacted criteria involved 1st instar mayflies in acute tests, or to begin chronic tests. A comparison of water quality criteria with the toxicity results from this study highlight four issues for the established criteria (Table 10). First, the results demonstrate that hardness plays an important role in chloride toxicity for mayflies, and most criteria do not account for hardness. Second, the acute criteria are often lower than the relevant LC50s for mayflies, but this may not be true for some species in soft water (e.g., *A. semirufum* or *P. fragile* in Spruce). Third, chronic criteria >200 mg Cl/L could be associated with measurable mayfly mortality (based on LC20s), especially in soft waters. Finally, the Iowa models that corrected for hardness produced acute criteria that appear to be relatively protective for all of the mayfly species and waters we examined except *A. semirufum* in soft (Spruce) water. However, the Iowa models that corrected for hardness produced chronic criteria that would appear to be associated with measureable mortality for all of the mayfly species and waters we examined.

Hardness was an important factor in both acute and chronic toxicity of chloride to the mayflies examined in this study, as was found in earlier studies of aquatic invertebrates (e.g., Mount et al. 1999, Soucek et al. 2011). Mayflies appear more sensitive to chloride in soft waters such as in Spruce relative to moderately hard or hard waters. Soft waters are relatively common in much of Pennsylvania (Figure 23), especially in watersheds where the underlying soils and bedrock do not have significant amounts of $CaCO_3$ (e.g., limestone). Among the soft-water streams, there are some where hardness is especially low (e.g., $\leq$ 20 mg/L), which may make the faunas in those streams more sensitive to chloride exposure than in other streams.

Table 10. Selected acute and chronic criteria for chloride, and LC50, LC50, and LC10 results from our study (see Tables 4, 5, and 6).

	Acute (mg/L)	Chronic (mg/L)	Chronic (mg/L)
PA (Ch 93)		250	potable water standard
EPA (1988)	860	230	
Nagpal et al. (2003) (for British Columbia)	600	150	
CCME (2011)	640	120	
From Iowa (2009)			
Default (200 mg/L)	629	389	
Based on hardness and sulfate	405-1010	250-624	
PA Soft [a]	382	236	
PA Moderately Hard [a]	597	369	
PA Hard [a]	703	435	
	LC50	LC20	LC10
N. triangulifer			
Soft	704	109	94
Moderately Hard	2141	175	140
Hard	1420	188	140
A. semirufum			
Soft	107	114	104
Moderately Hard	1827	279	235
Hard	1336	128	102
P. fragile			
Soft	472	168	150
Moderately Hard	2110	332	303
Hard	1765	245	210
E. invaria			
Soft	2016	-	-
Moderately Hard	4500	-	-
Hard	4762	-	-
L. cupida			
Soft	4667	-	-
Moderately Hard	5921	-	-
Hard	5832	-	-
M. modestum			
Soft	2065	-	-
Moderately Hard	2763	138 (WCC)	133 (WCC)
Hard	4329	-	-

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[a] References to Soft (6 mg/L; Spruce), Moderately Hard (94 mg/L; House), and Hard (212 mg/L; Cedar) waters are specific to the Pennsylvania waters included in these experiments.

[b] For Iowa Criteria, acute formula = $287.8(\text{hardness})^{0.205797}(\text{sulfate})^{-0.07452}$, and chronic formula = $177.87(\text{hardness})^{0.205797}(\text{sulfate})^{-0.07452}$

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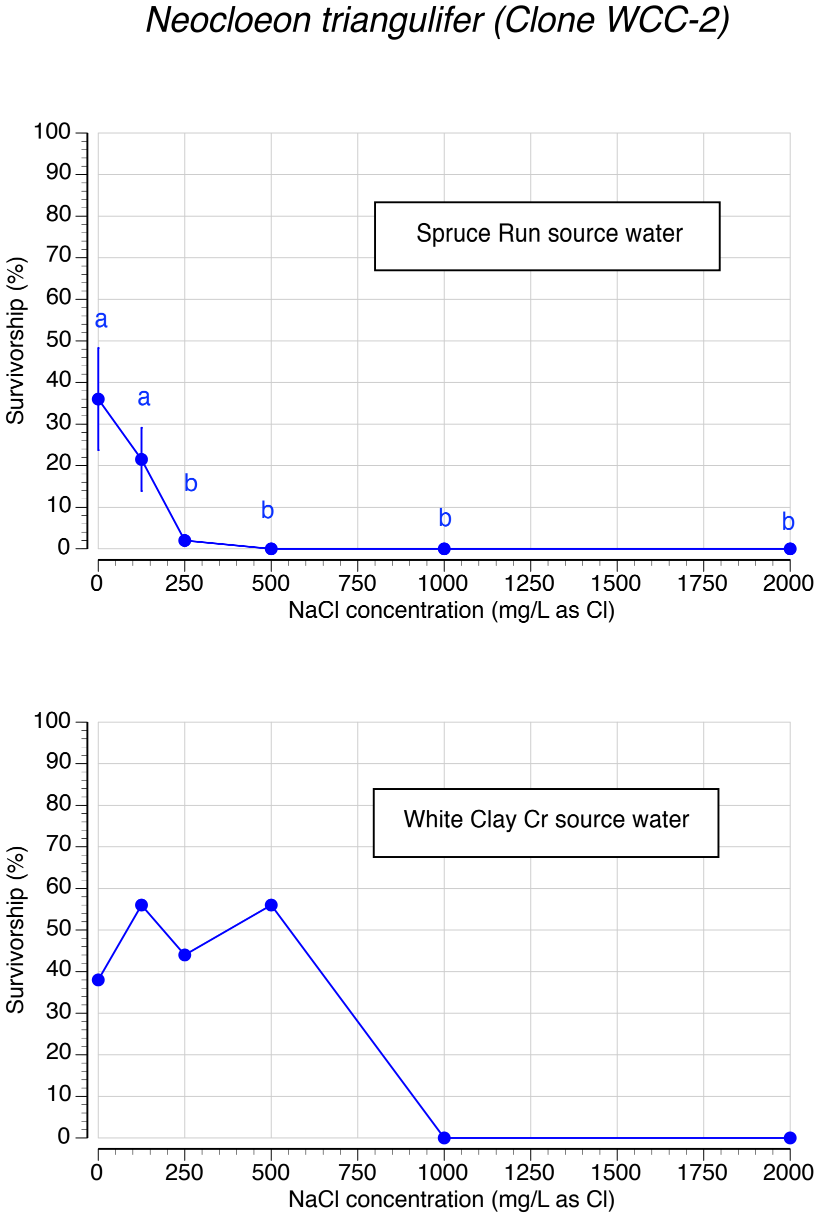

Figure 1. Lifecycle survivorship (1^{st} instar to adult, %) of *Neocloeon triangulifer* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Spruce Run and White Clay Creek.

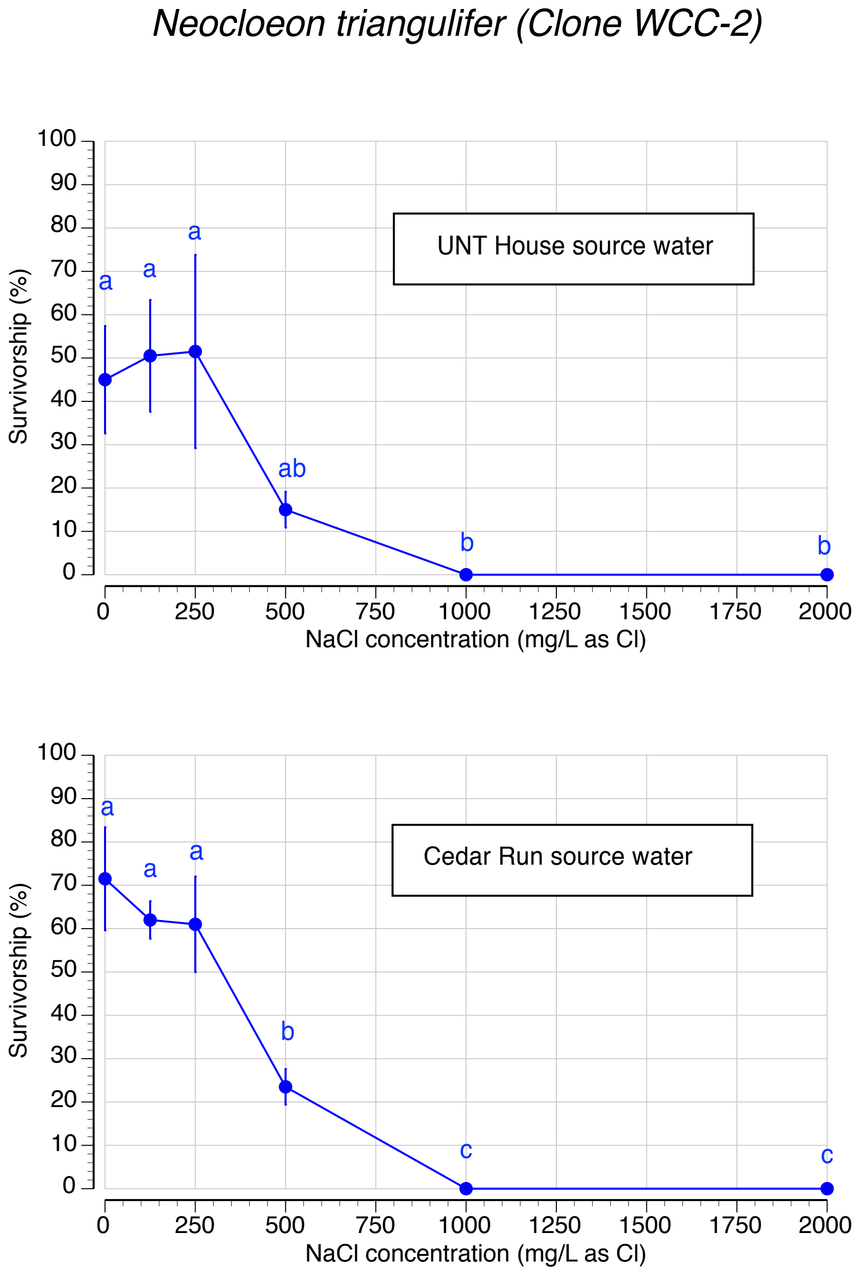

Figure 2. Lifecycle survivorship (1st instar to adult, %) of *Neocloeon triangulifer* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from unnamed tributary to House Run and Cedar Run.

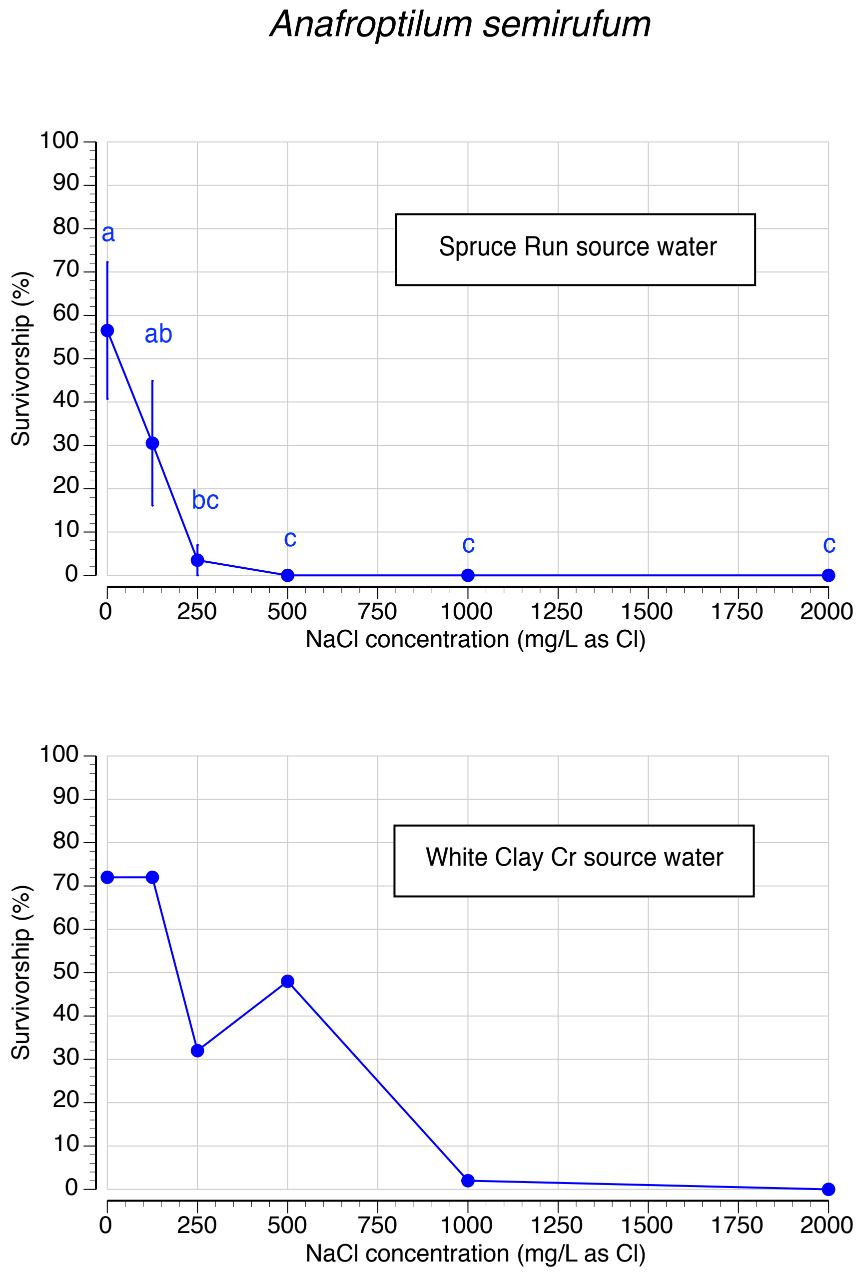

Figure 3. Lifecycle survivorship (1st instar to adult, %) of *Anafroptilum semirufum* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Spruce Run and White Clay Creek.

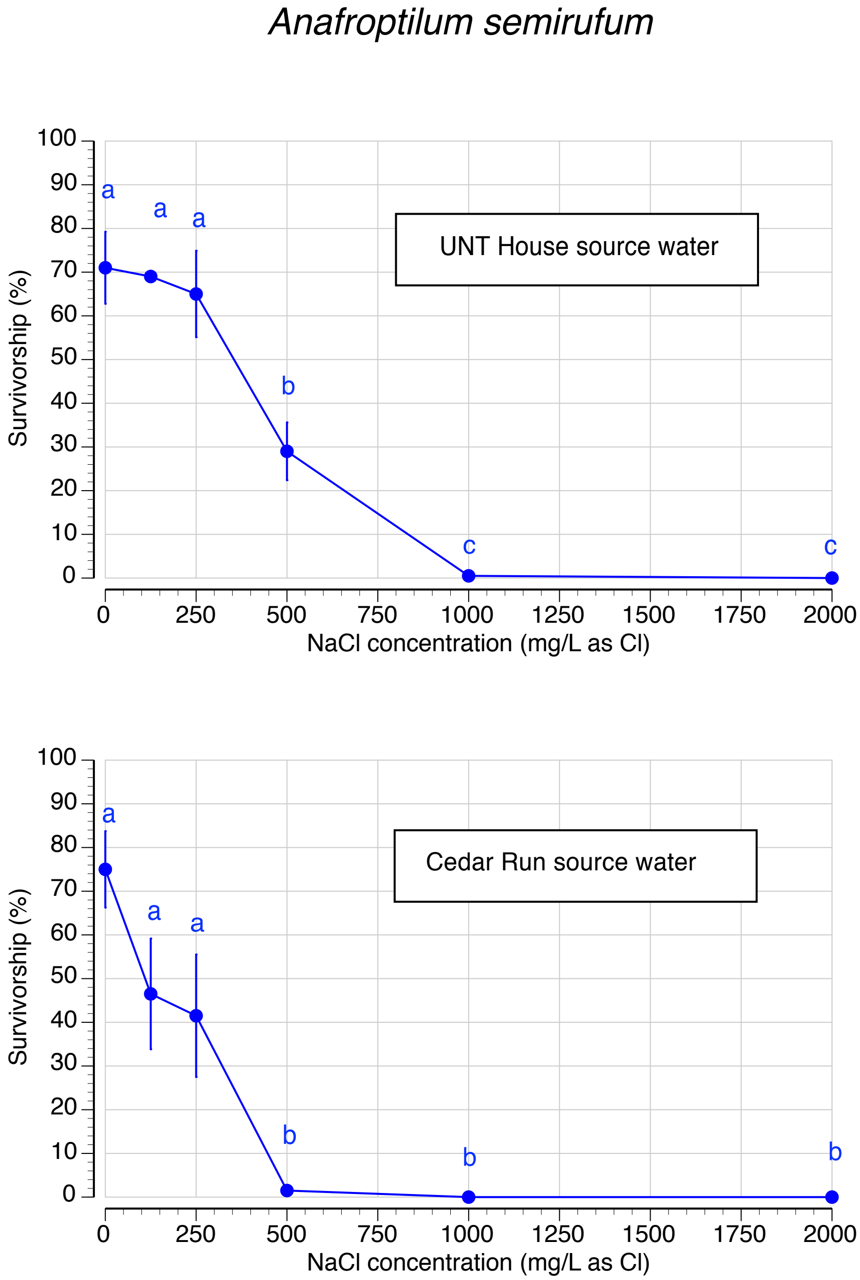

Figure 4. Lifecycle survivorship (1st instar to adult, %) of *Anafroptilum semirufum* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from unnamed tributary to House Run and Cedar Run.

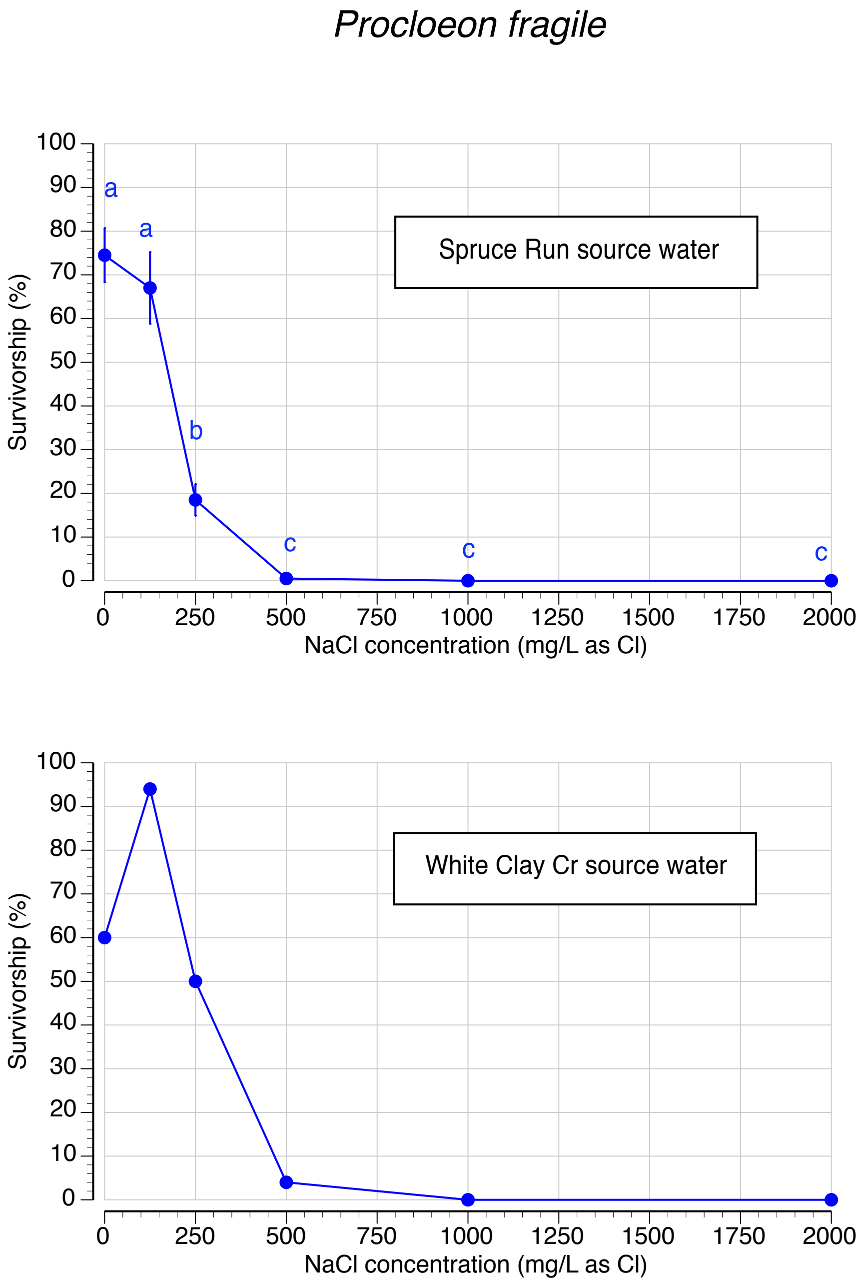

Figure 5. Lifecycle survivorship (1st instar to adult, %) of *Procloeon fragile* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Spruce Run and White Clay Creek.

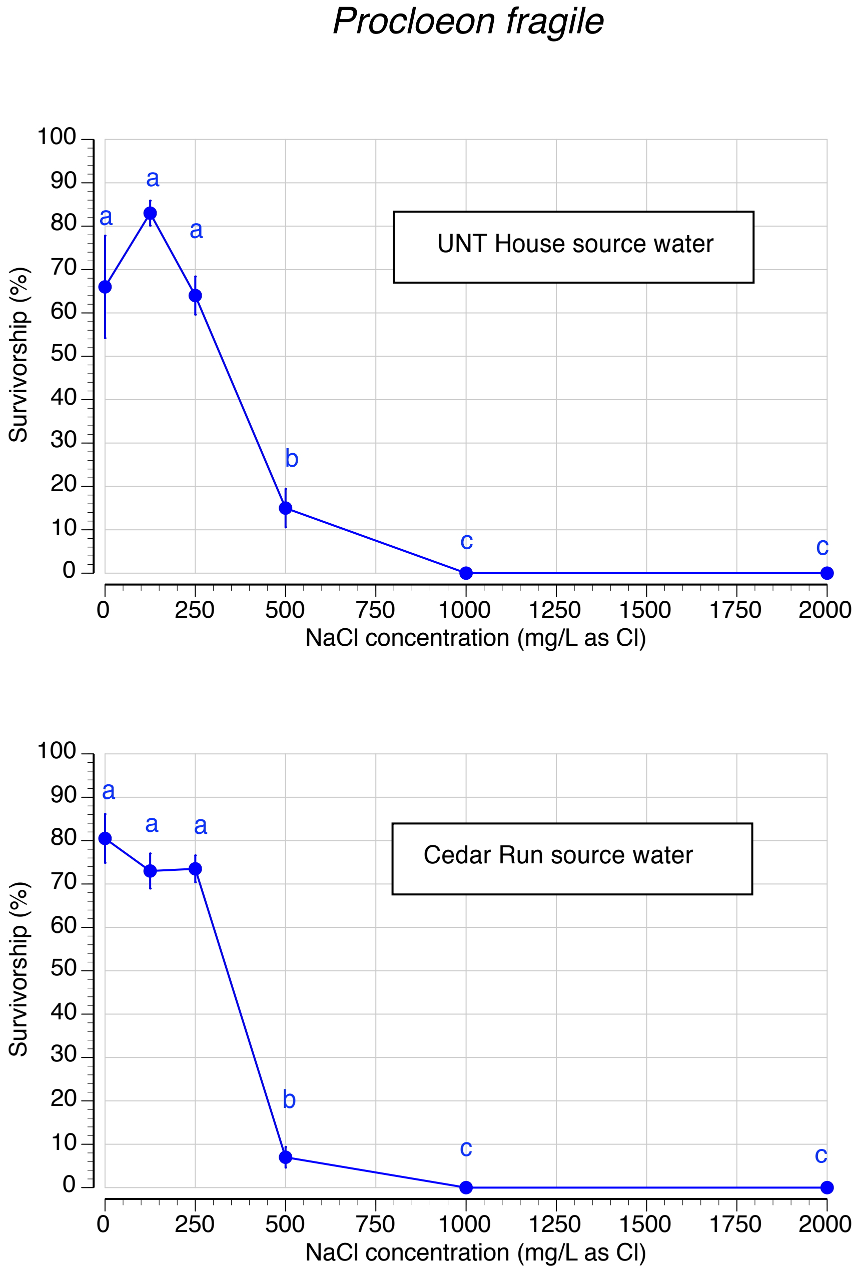

Figure 6. Lifecycle survivorship (1st instar to adult, %) of *Procloeon fragile* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from unnamed tributary to House Run and Cedar Run.

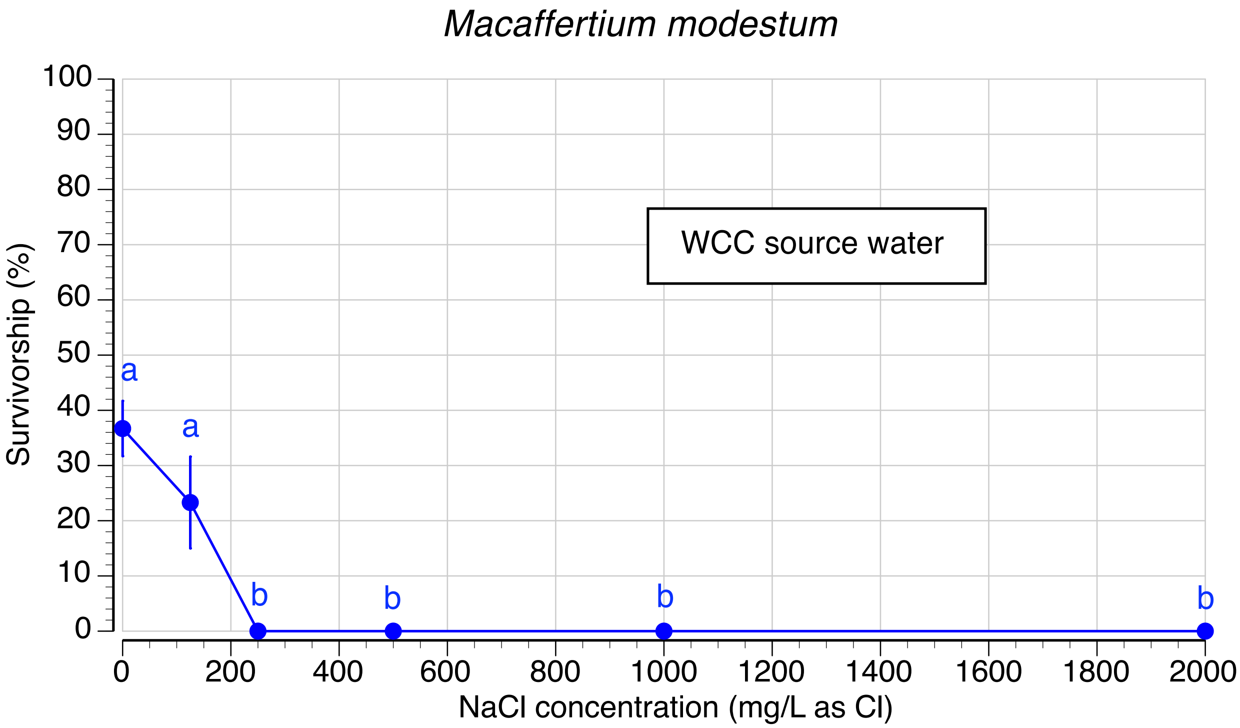

Figure 7. Chronic (50 d) survivorship (1st instar to adult, %) of *Macaffertium modestum* in tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from White Clay Creek.

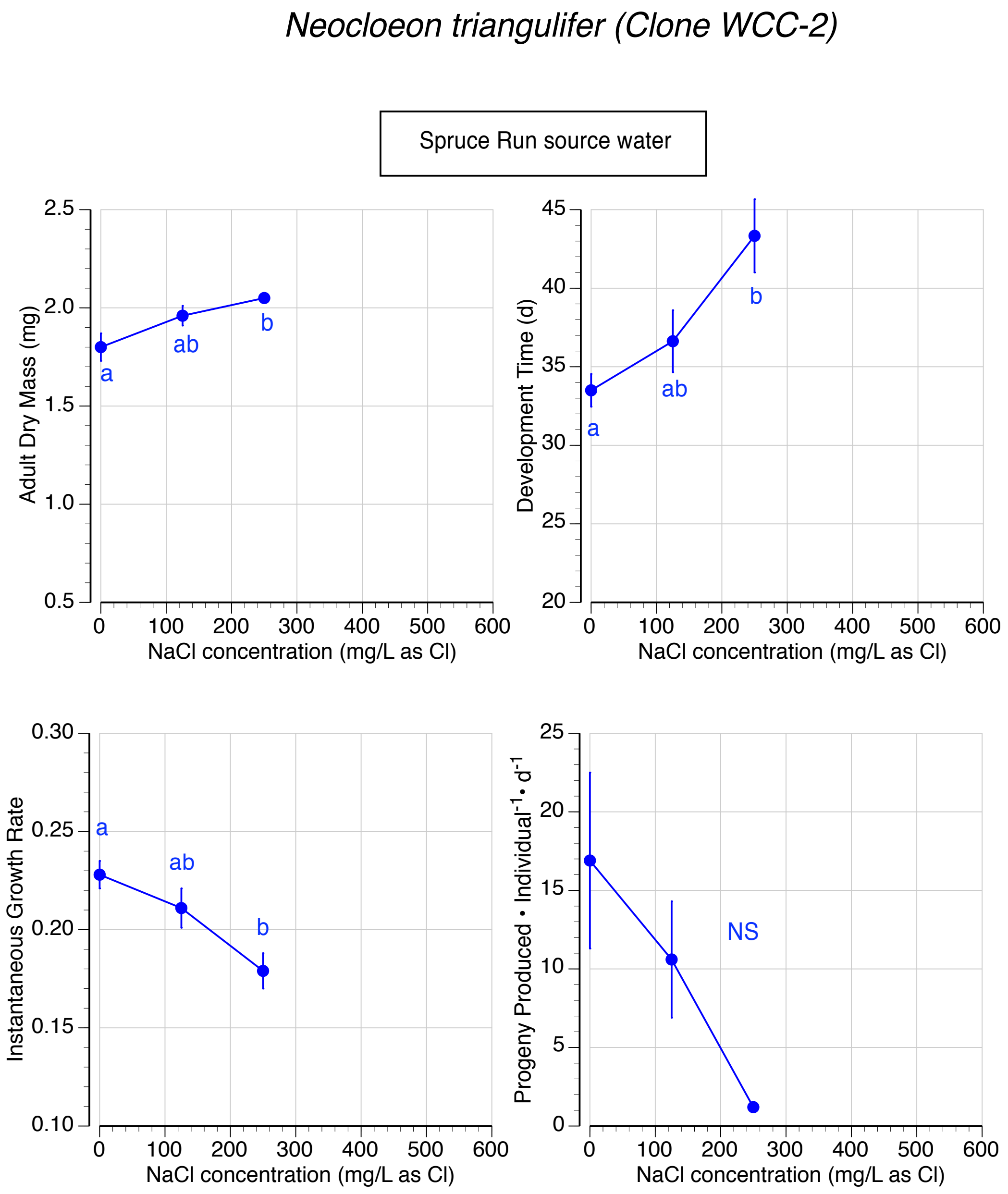

Figure 8. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) of *Neocloeon triangulifer* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Spruce Run. There were no survivors in the highest three concentrations.

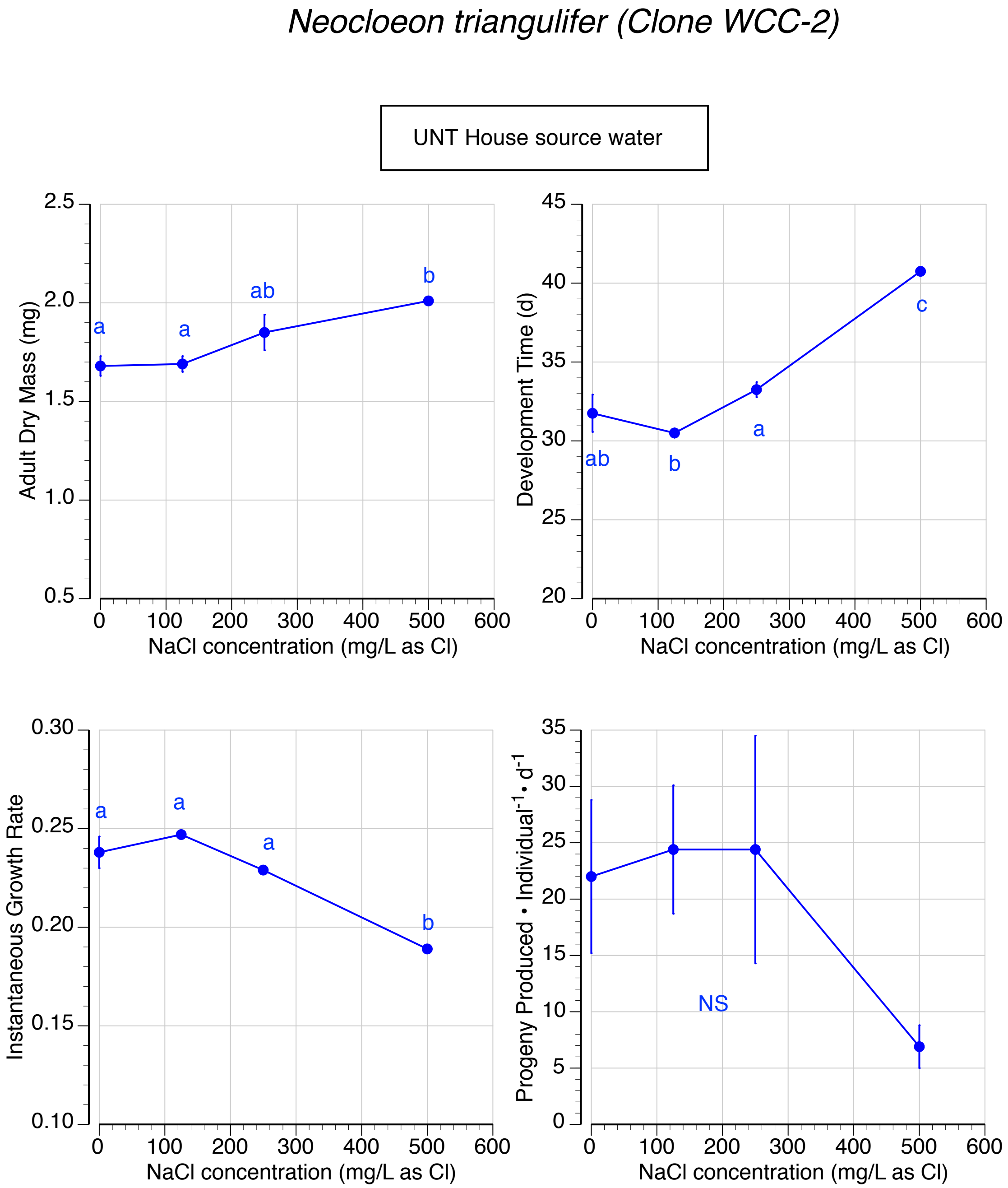

Figure 9. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) of *Neocloeon triangulifer* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from unnamed tributary to House Run. There were no survivors in the highest two concentrations.

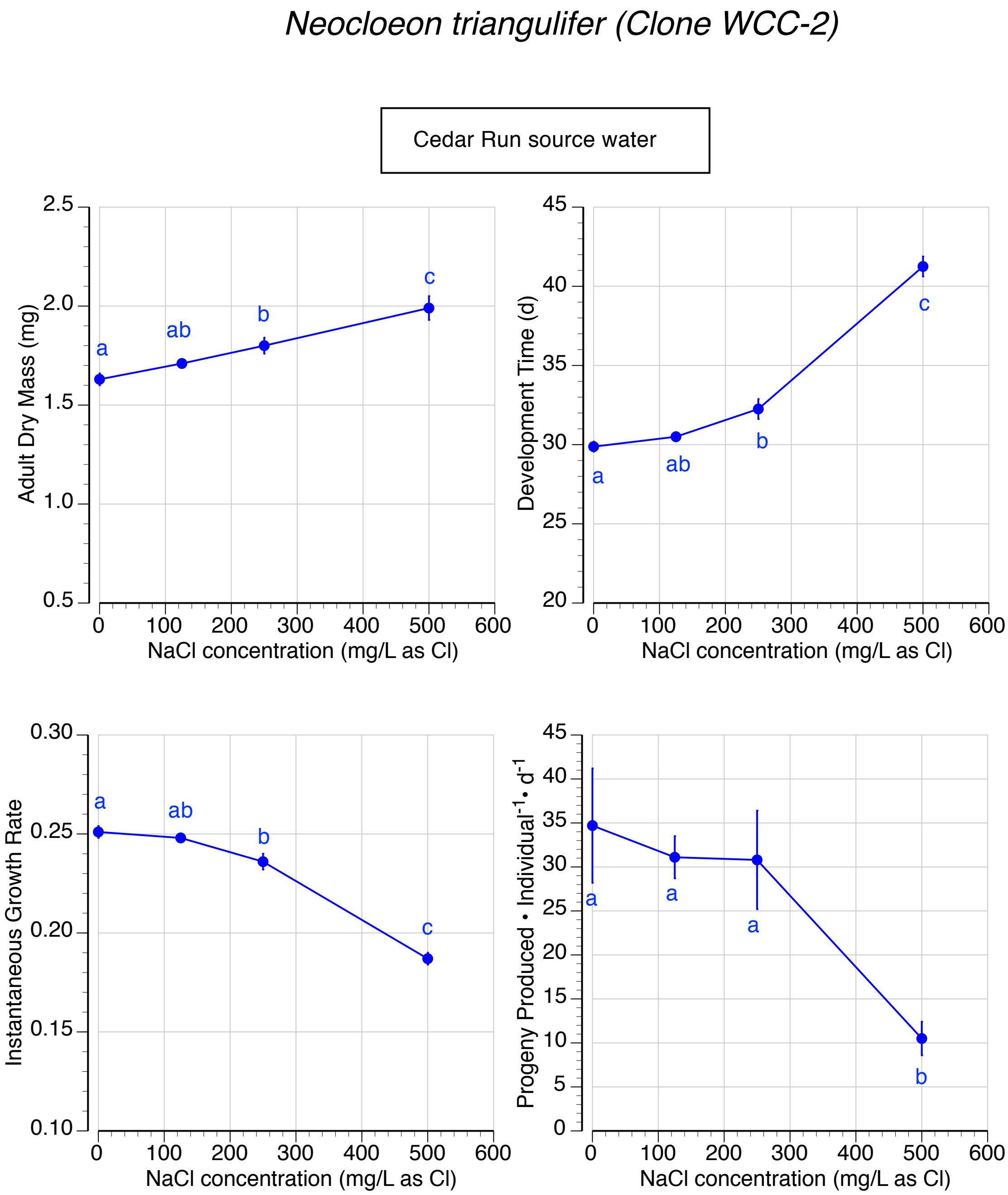

Figure 10. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) of *Neocloeon triangulifer* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Cedar Run. There were no survivors in the highest two concentrations.

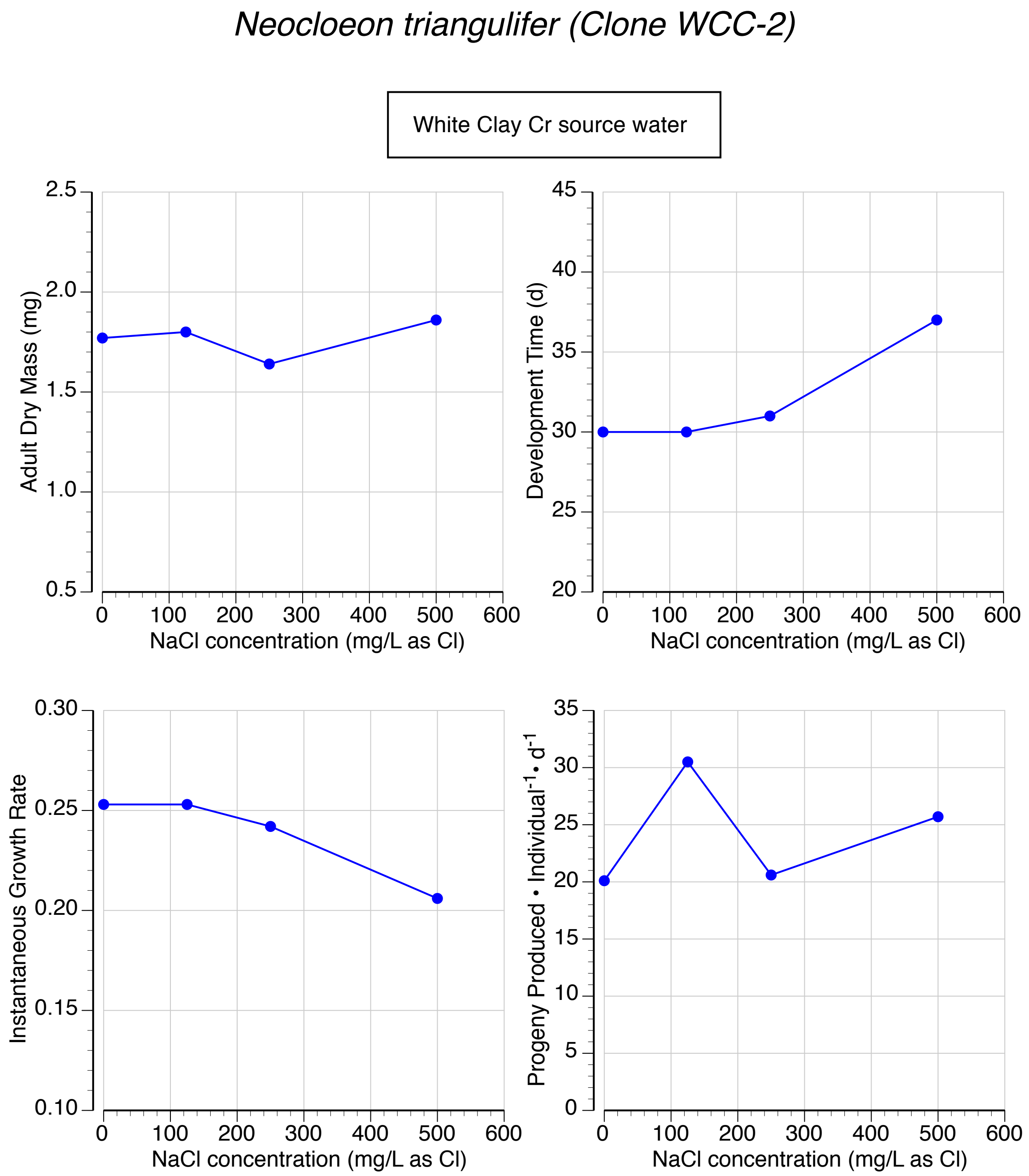

Figure 11. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) of *Neocloeon triangulifer* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from White Clay Creek. There were no survivors in the highest two concentrations.

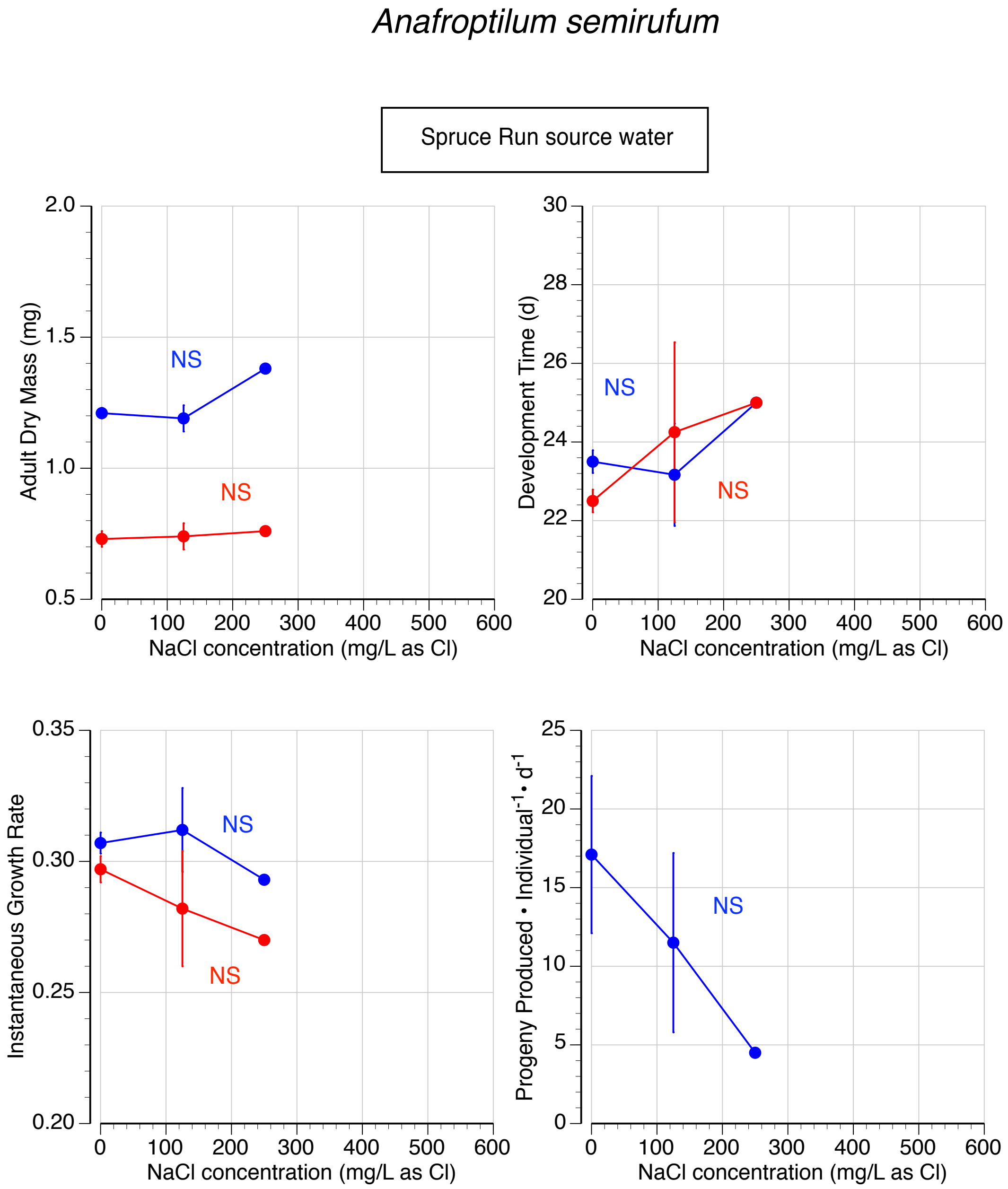

Figure 12. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Anafroptilum semirufum* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Spruce Run. There were no survivors in the highest three concentrations.

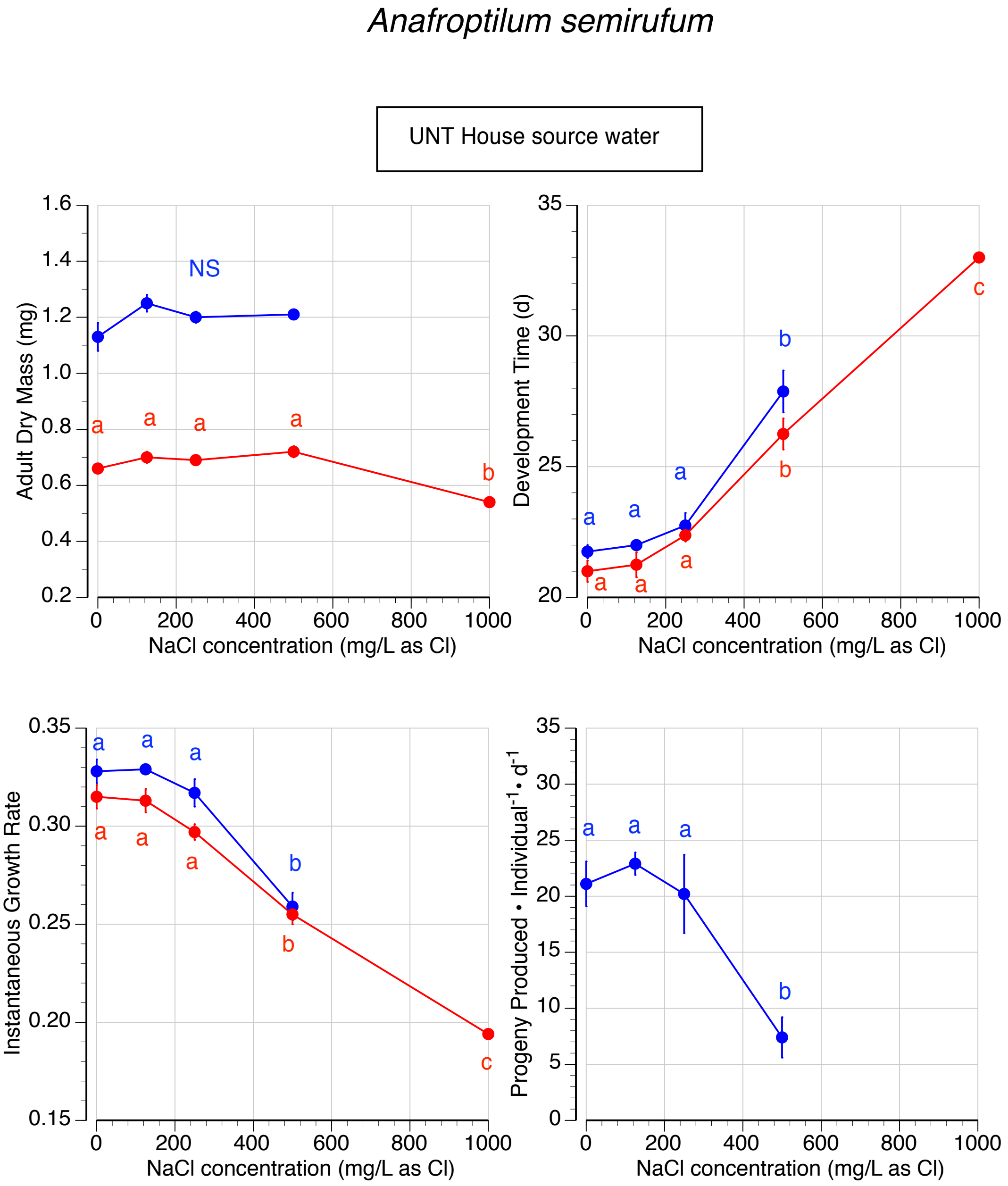

Figure 13. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Anafroptilum semirufum* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from unnamed tributary to House Run. There were no survivors in the highest two concentrations.

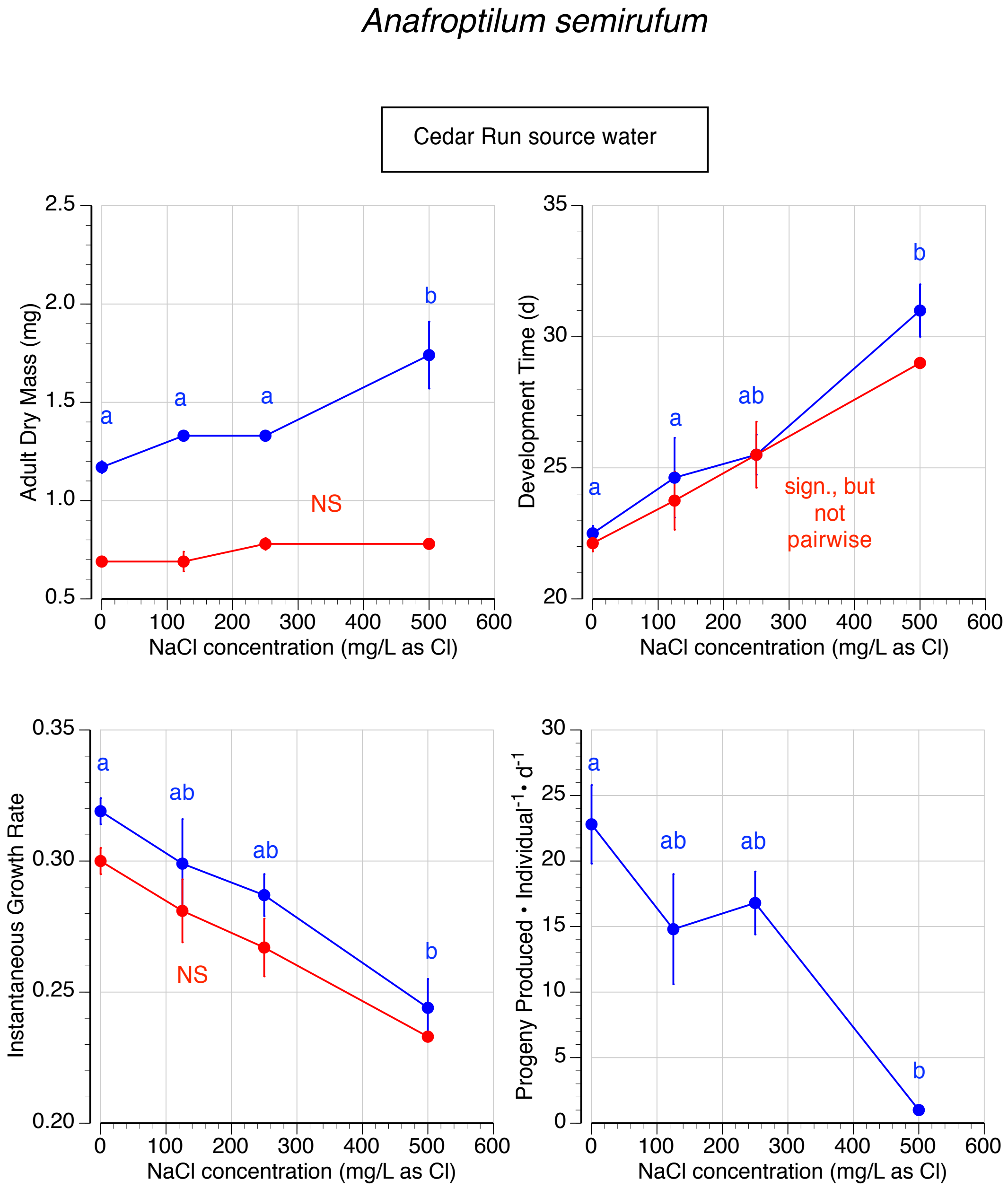

Figure 14. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Anafroptilum semirufum* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Cedar Run. There were no survivors in the highest two concentrations.

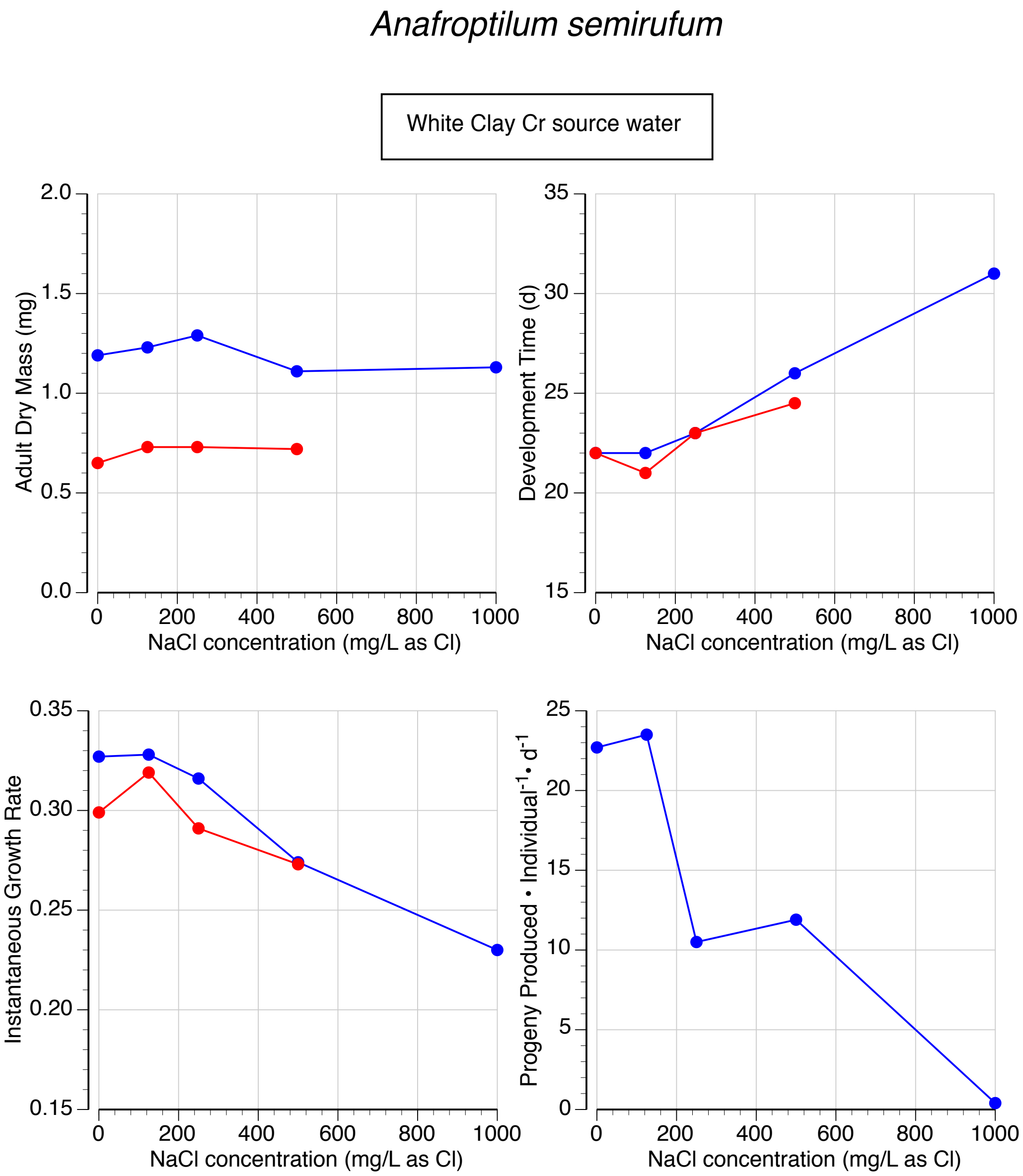

Figure 15. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Anafroptilum semirufum* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from White Clay Creek. There were no male survivors in 1000 and 2000 mg Cl/L concentrations, and no female survivors in 2000 mg Cl/L concentration.

Procloeon fragile

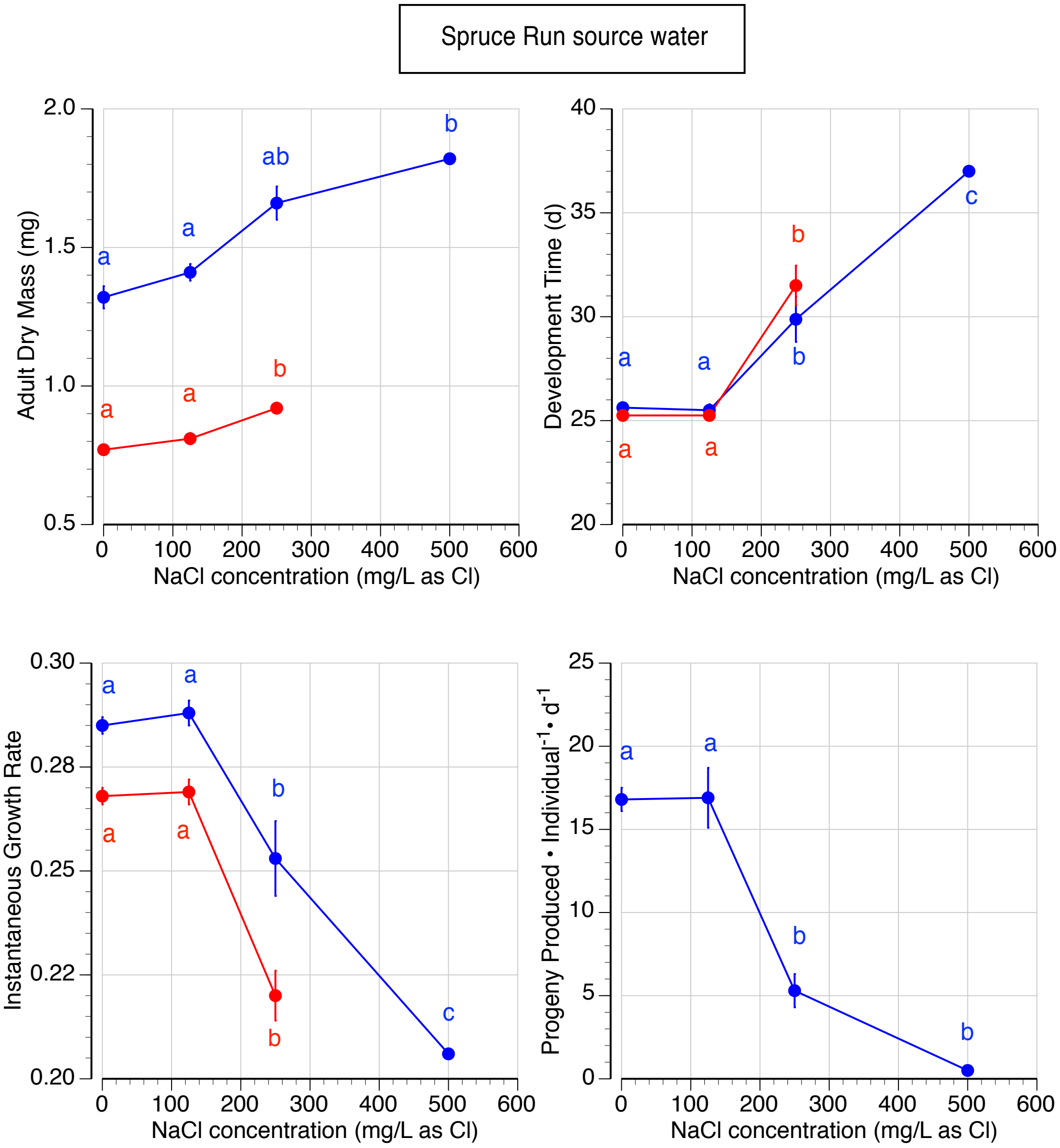

Figure 16. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Procloeon fragile* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Spruce Run. There were no survivors in the highest three concentrations.

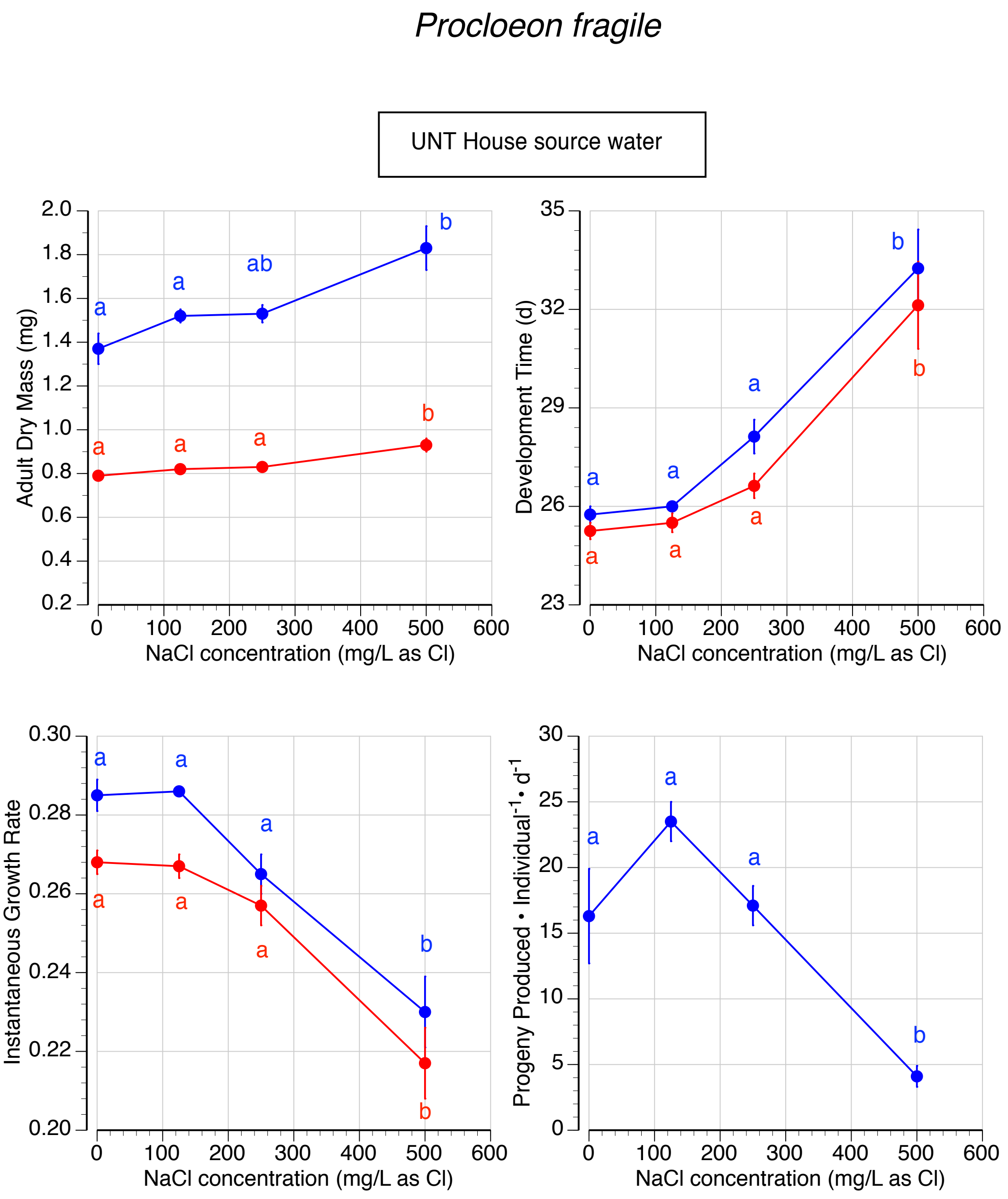

Figure 17. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Procloeon fragile* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from unnamed tributary to House Run. There were no survivors in the highest two concentrations.

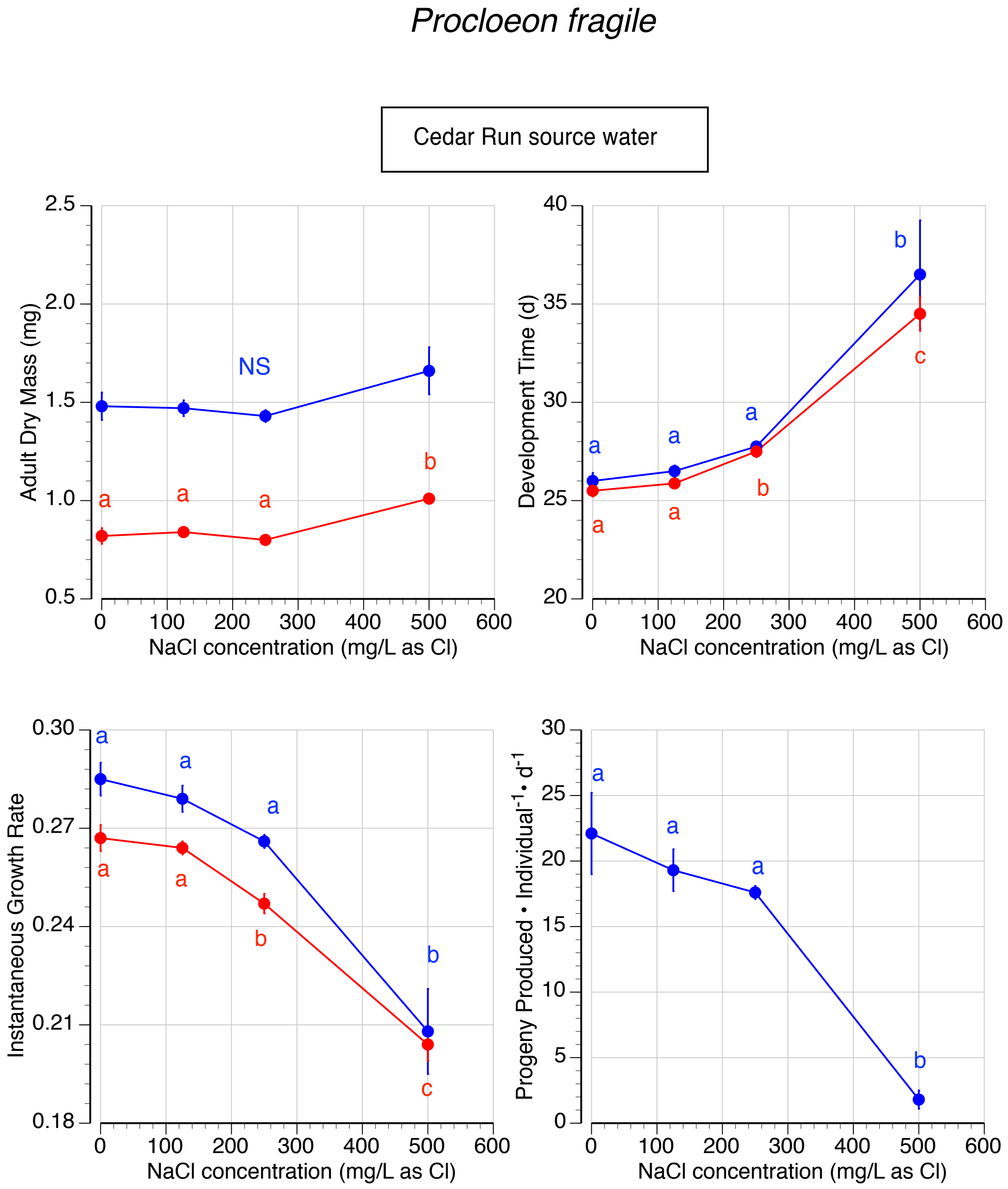

Figure 18. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Procloeon fragile* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from Cedar Run. There were no survivors in the highest two concentrations.

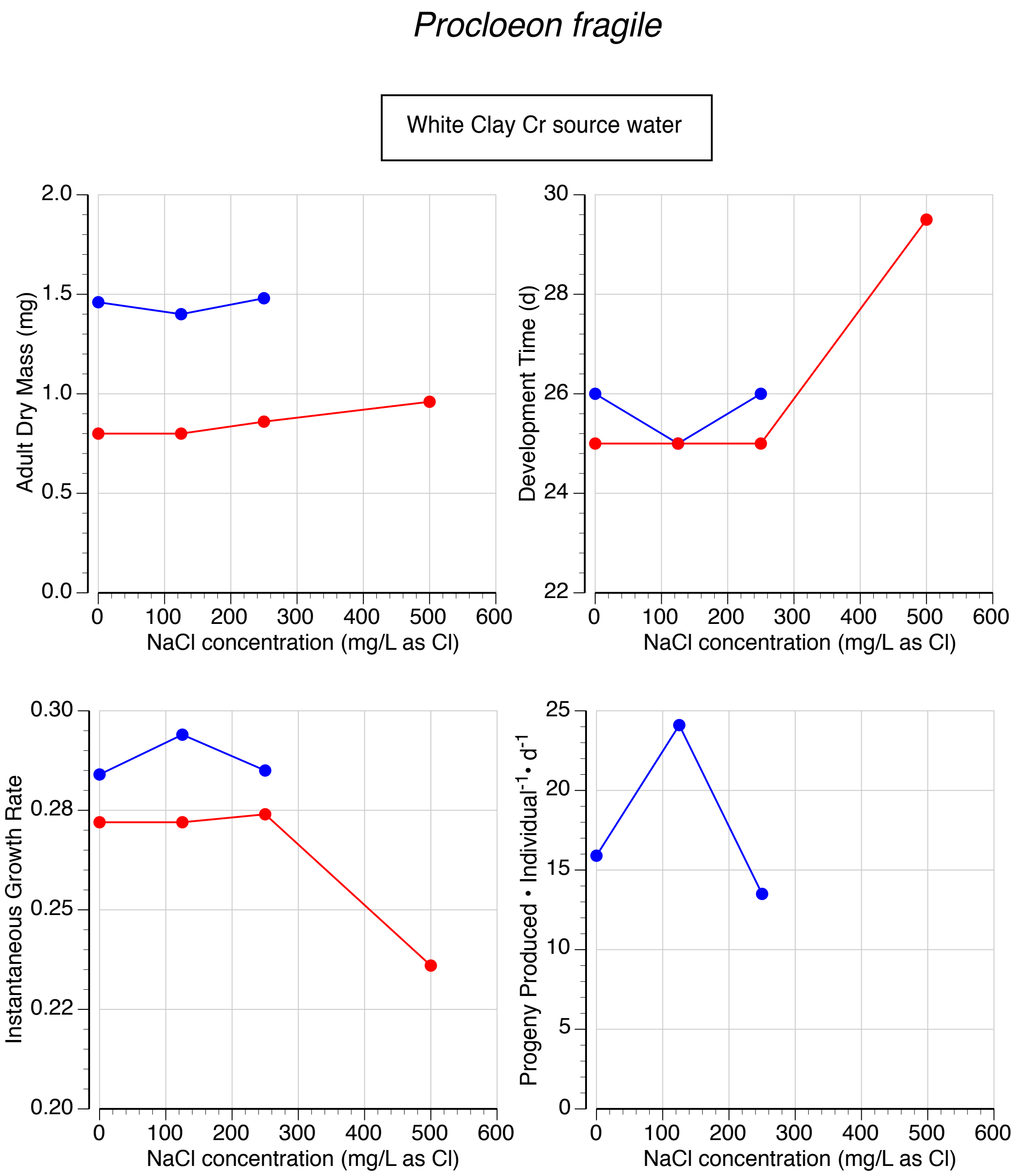

Figure 19. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate (IGR) and population growth rate (PGR; progeny produced per individual per day) for females (blue) and males (red) of *Procloeon fragile* in chronic tests using NaCl (0, 125, 250, 500, 1000 and 2000 mg Cl/L) dissolved in water from White Clay Creek. There were no male survivors in 1000 and 2000 mg Cl/L concentrations, and no female survivors in 2000 mg Cl/L concentration.

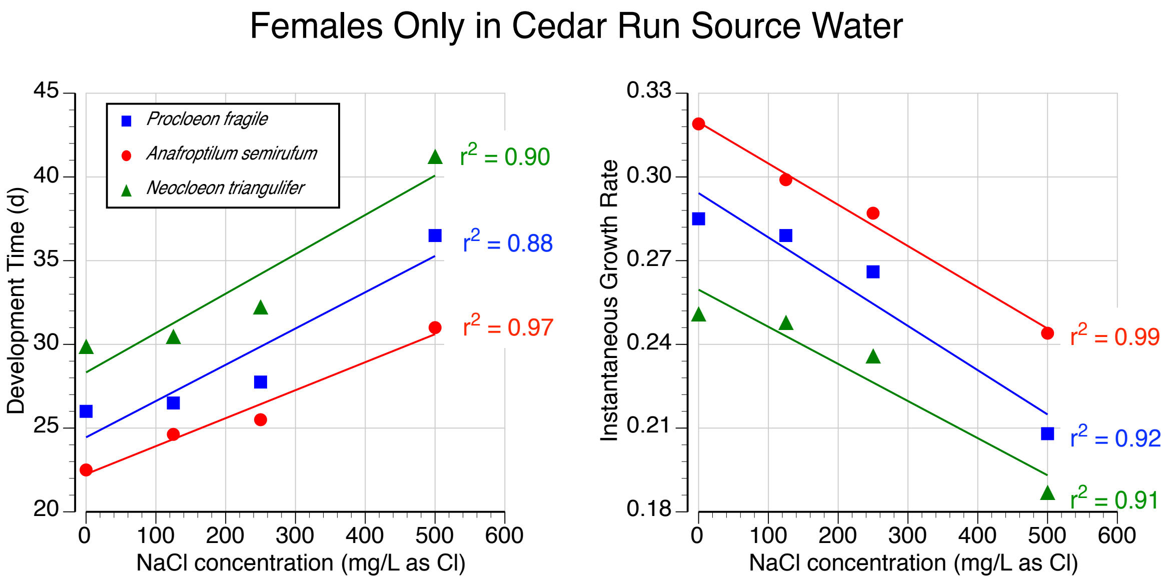

Figure 20. Larval development time (d) and instantaneous growth rate (IGR) for female survivors of *N. triangulifer*, *A. semirufum*, and *P. fragile* in chronic tests using NaCl (0, 125, 250, 500 mg Cl/L) dissolved in water from Cedar Run. There were no survivors in 1000 and 2000 mg Cl/L concentrations.

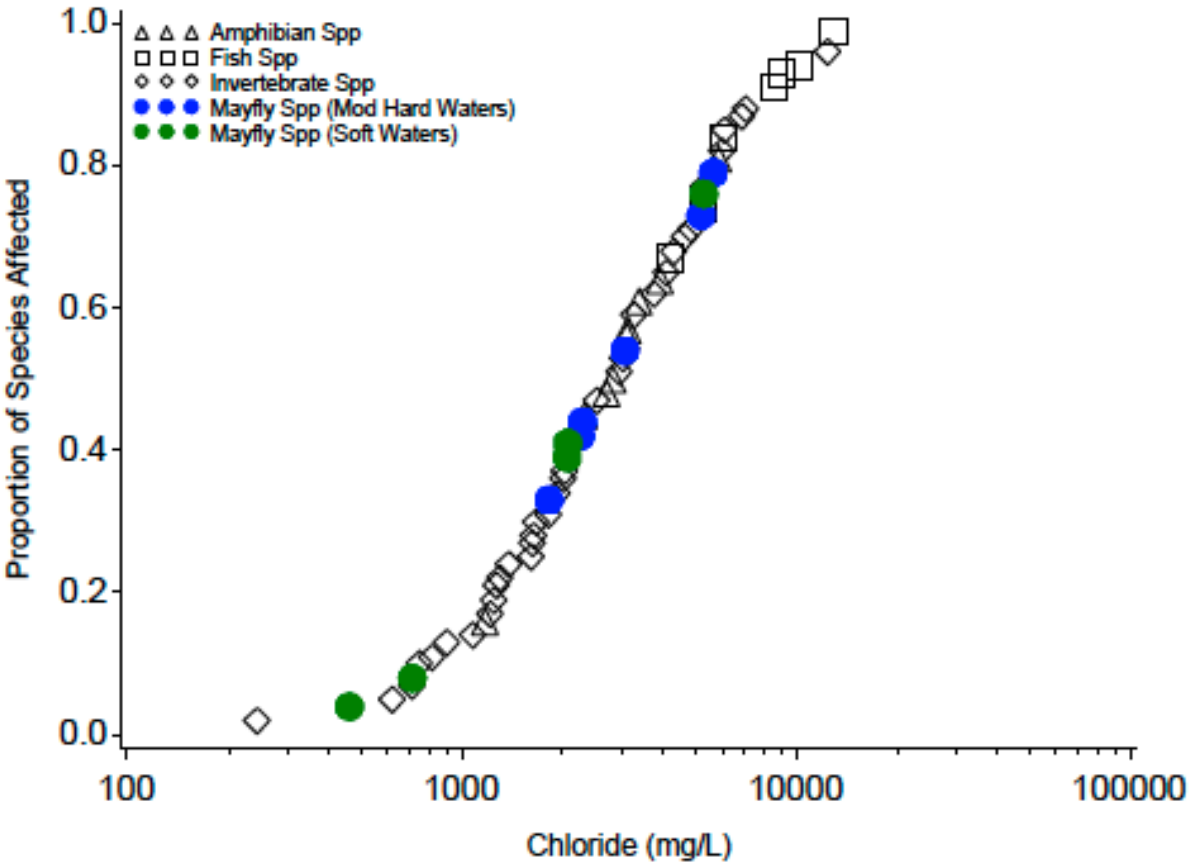

Figure 21. Short-term LC50 data for six mayflies in moderately hard (House) and soft (Spruce) waters plotted in reconstruction of Fig. 3 (CCME 2011) showing acceptable toxicity data for 53 aquatic species versus Hazen plotting position (proportion of species affected).

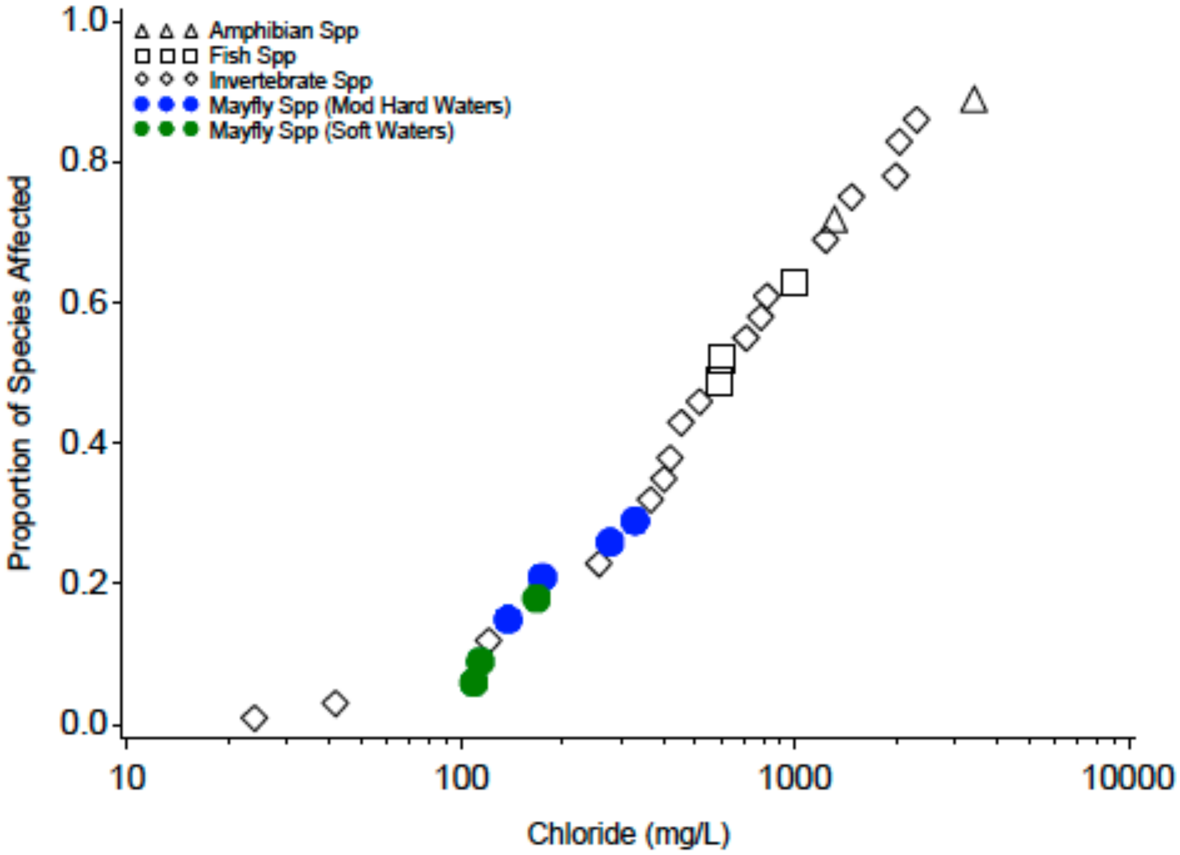

Figure 22. Long-term LC 20 toxicity data for three mayflies in moderately hard (House) and soft (Spruce) waters plotted in a reconstruction of Fig. 5 (CCME 2011) showing acceptable toxicity data for 28 aquatic species versus Hazen plotting position (proportion of species affected).

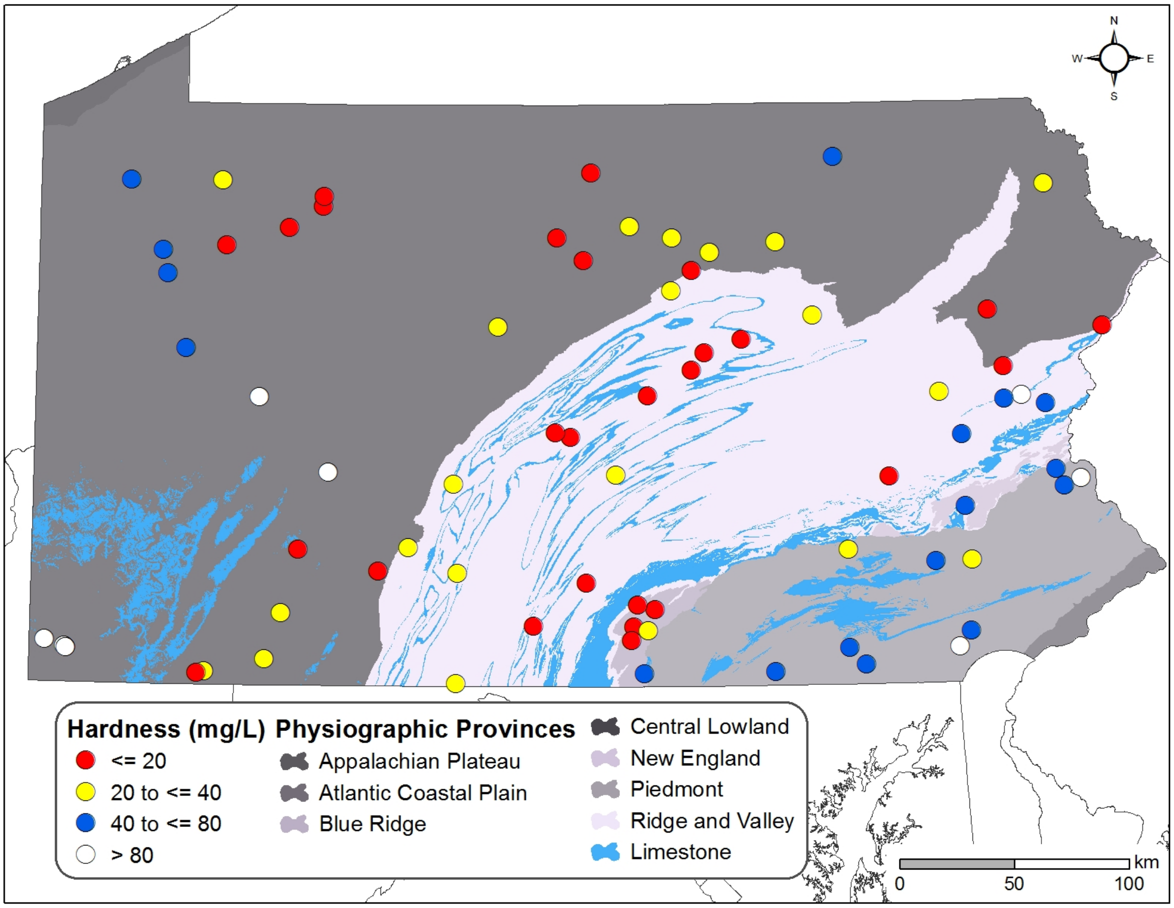

Figure 23. Plot of four categories of hardness based on data provided by PADEP for PADEP regional reference sites and WQN reference sites across Pennsylvania.

Appendix 1. Chemical characteristics of source waters on three sample dates in 2014.

		Spruce			UNT House			Cedar			WCC		
		29-Apr	2-Jul	3-Sep	29-Apr	2-Jul	3-Sep	29-Apr	2-Jul	3-Sep	29-Apr	2-Jul	3-Sep
pH, Lab (Electrometric)	pH units	6.6	6.9	6.8	7.9	8.1	7.9	8.6	8.5	8.3	7.5	7.8	7.8
Alkalinity @ pH 4.5	mg/L	4.2	3.6	4.4	75.4	83.4	101	176.8	188.4	183	60.2	61.8	67
Total Chloride-Ion Chromatograph	mg/L	<0.5	0.56	0.69	1.18	1.17	1.24	12.6	12.22	13.43	12.06	11.18	9.45
Total Sulfate-Ion Chromatograph	mg/L	3.9	3.41	3.5	19.5	14.4	13.16	16.38	16.57	16.52	17.64	17.37	16.51
Specific Conductivity @ 25.0 C	µS/cm	18.55	18.14	18.26	190.5	189.4	231	412	432	422	220	230	232
Hardness, Total (Calculated)	mg/L	6	7	6	89	86	107	206	218	212	88	91	89
Calcium, Total (Water & Waste) by ICP	mg/L	1.324	1.487	1.293	28	26.8	33.8	51.181	53.6	51.7	20.8	22.4	22.4
Magnesium, Total (Water & Waste) by ICP	mg/L	0.725	0.779	0.678	4.673	4.713	5.417	18.895	20.5	20.1	8.669	8.531	7.95
Sodium, Total (Water & Waste) by ICP	mg/L	0.426	0.399	0.635	3.23	2.881	4.052	5.131	5.266	5.482	6.358	6.167	6.937
Potassium, Total (Water & Waste) by ICP	mg/L	<1	<1	<1	1.142	1.909	2.846	1.331	1.232	1.355	1.691	1.914	2.468
Total Dissolved Solids @ 180C by USGS-I-1750	mg/L	26	20	30	122	142	148	238	274	252	152	no data	166

Appendix 2. Experimental details for acute (48 h) toxicity tests involving 1st instar larvae for six mayfly species, four source waters of differing hardness, and six concentrations (five chloride concentrations and one control with no chloride added; Table 3).

Test Animals	Source Waters	No. Cl Dilutions	No. Tests	No. Reps	No. Animals/ Vessel	Test Duration	Water Collection Date
Neocloeon triangulifer	Spruce (soft)	5+ctrl	4	1	20	48h	29 Apr
	House (med)	5+ctrl	4	1	20	48h	29 Apr
	Cedar (hard)	5+ctrl	4	1	20	48h	29 Apr
	WCC (med)	5+ctrl	4	1	20	48h	29 Apr
Anafroptilum semirufum	Spruce (soft)	5+ctrl	4	1	20	48h	2 Jul
	House (med)	5+ctrl	4	1	20	48h	2 Jul
	Cedar (hard)	5+ctrl	4	1	20	48h	2 Jul
	WCC (med)	5+ctrl	4	1	20	48h	2 Jul
Procloeon fragile	Spruce (soft)	5+ctrl	4	1	20	48h	3 Sep
	House (med)	5+ctrl	4	1	20	48h	3 Sep
	Cedar (hard)	5+ctrl	4	1	20	48h	3 Sep
	WCC (med)	5+ctrl	4	1	20	48h	3 Sep
Macaffertium modestum	Spruce (soft)	5+ctrl	4	1	20	48h	2 Jul
	House (med)	5+ctrl	4	1	20	48h	2 Jul
	Cedar (hard)	5+ctrl	4	1	20	48h	2 Jul
	WCC (med)	5+ctrl	4	1	20	48h	2 Jul
Leptophlebia cupida	Spruce (soft)	5+ctrl	4	1	20	48h	29 Apr
	House (med)	5+ctrl	4	1	20	48h	29 Apr
	Cedar (hard)	5+ctrl	4	1	20	48h	29 Apr
	WCC (med)	5+ctrl	4	1	20	48h	29 Apr
Ephemerella invaria	Spruce (soft)	5+ctrl	4	1	20	48h	2 Jul
	House (med)	5+ctrl	4	1	20	48h	2 Jul
	Cedar (hard)	5+ctrl	4	1	20	48h	2 Jul
	WCC (med)	5+ctrl	4	1	20	48h	2 Jul

Measurements: larval survival (%)
Endpoints: LC50

Appendix 3. Experimental details for chronic (whole lifecycle) toxicity tests involving three mayfly species, four source waters, and six concentrations (five chloride concentrations and one control; Table 3).

Test Animals	Source Waters	No. Dilutions	No. Tests	No. Reps	No. Animals/ Vessel	Test Duration	Water Collection Date
Neocloeon triangulifer	Spruce (soft)	5+ctrl	4	1	50	20–40d	29 Apr
	House (med)	5+ctrl	4	1	50	20–40d	29 Apr
	Cedar (hard)	5+ctrl	4	1	50	20–40d	29 Apr
	WCC (med)	5+ctrl	4	1	50	20–40d	29 Apr
Anafroptilum semirufum	Spruce (soft)	5+ctrl	4	1	50	20–40d	2 Jul
	House (med)	5+ctrl	4	1	50	20–40d	2 Jul
	Cedar (hard)	5+ctrl	4	1	50	20–40d	2 Jul
	WCC (med)	5+ctrl	4	1	50	20–40d	2 Jul
Procloeon fragile	Spruce (soft)	5+ctrl	4	1	50	20–40d	3 Sep
	House (med)	5+ctrl	4	1	50	20–40d	3 Sep
	Cedar (hard)	5+ctrl	4	1	50	20–40d	3 Sep
	WCC (med)	5+ctrl	4	1	50	20–40d	3 Sep
Macaffertium modestum	Spruce (soft)	5+ctrl	2	1	50	50d	3 Sep
	WCC (med)	5+ctrl	2	1	50	50d	3 Sep

Measurements: larval survival, larval development time (d), adult dry mass, Instantaneous Growth Rate (IGR), Population Growth Rate (PGR)

Endpoints: NOEC, LOEC, LC50, LC20, LC10, EC20