

Weak differences in sensitivity to major ions by different larval stages of the mayfly *Neocloeon triangulifer*

Sarah E. Orr^{1,2}, Jamie K. Cochran^{1,3}, Ian G. Wallace^{1,4}, Rachel W. Gray^{1,5}, Gretta E. Overmyer^{1,6}, and David B. Buchwalter^{1,7}

¹Department of Biological Sciences, North Carolina State University, 850 Main Campus Drive, Raleigh, North Carolina 27606 USA

Abstract: Freshwater salinization is a global ecological concern because of the alarming biodiversity declines associated with increases in major ion concentrations. Loss of mayfly diversity appears to be a common ecological response to anthropogenic salinization worldwide. Remarkably few regulatory standards exist to protect aquatic life from major ions, and antiquated approaches for setting such standards rely on traditional laboratory toxicity tests, which do not address sensitivities of mayflies at different larval stages. The lab-reared mayfly *Neocloeon triangulifer* (McDunnough, 1931) has emerged as one of the very few useful aquatic insect models for studying the effects of environmental stressors, including salinity, in the laboratory. Here, we asked if different larval life stages are differentially sensitive to ion concentrations by conducting traditional 96-h toxicity tests with NaCl, CaCl₂, and Ca/MgSO₄. We used a general linear model to determine if survivorship differed among larval stages as well as ion type and concentration. We also calculated median lethal concentrations (LC₅₀) for each larval stage. Larval sensitivity to NaCl decreased slightly with age (2–6, 9–13, and 17–21 d, with LC₅₀ values of 401, 441, and 570 mg/L, respectively, when expressed as Na concentrations). Similarly, larval sensitivity to Ca/MgSO₄ differed slightly among age groups (LC₅₀ = 748, 1503, and 1439 mg/L, respectively, when expressed as SO₄ concentrations). Reliable confidence intervals on LC₅₀ values for CaCl₂ could not be calculated because of high survivorship. However, our general linear model revealed that age played a moderate role in survival ($p = 0.0065$) across all salts of interest. To assess the potential changes in ion flux between larval stages, we used radiotracers (²²Na, ³⁵SO₄, or ⁴⁵Ca) in 18- and 25-d-old larvae and found no strong differences in ion uptake rates. We also qualitatively examined morphological differences between larval life stages, including the appearance of gills and number of ionocytes. Our results indicate that younger *N. triangulifer* larvae may be more sensitive to major ions than mature larvae. These results should be considered when experimentally using larger, late-stage *N. triangulifer* larvae to study the physiological effects and acute toxicity of salinity.

Key words: salinity, aquatic insects, mayflies, ontogenetic, major ions, toxicity, freshwater salinization, life stage sensitivity, model organism

Many freshwater ecosystems are becoming saltier worldwide (Canedo-Arguelles et al. 2016, Kaushal et al. 2018). Anthropogenic activities such as resource extraction, irrigation runoff, and road deicing are the main contributors to salt pollution in freshwaters (Pond et al. 2008, Entrekin et al. 2011, Kefford et al. 2016). However, drought and seawater intrusions also play a role in altering major ion concentrations in freshwater ecosystems (Kinzelbach et al. 2003, Barlow and Reichard 2010, Mosley 2017). Ecologists have observed sensitive aquatic organisms disappearing in affected

areas (Pond et al. 2008, Entrekin et al. 2011), but mitigation is unlikely without governmental water quality standards for salinity.

The only current federal water quality criteria for salinity in the United States is for a single ion, chloride (USEPA 1988), which does not sufficiently protect aquatic life (Pond et al. 2008). Because water-quality criteria still rely on outdated approaches, such as an amalgamation of single-species tests (Stephan et al. 1985), it is important to broaden the array of suitable test organisms to address specific ecological

E-mail addresses: ²seorr@ncsu.edu; ³jkcochra@ncsu.edu; ⁴iwallac@ncsu.edu; ⁵rwgray@ncsu.edu; ⁶geovermy@ncsu.edu; ⁷To whom correspondence should be addressed, dbbuchwa@ncsu.edu

Received 29 September 2021; Accepted 10 December 2021; Published online 21 February 2022. Associate Editor, Will Clements.

Freshwater Science, volume 41, number 2, June 2022. © 2022 The Society for Freshwater Science. All rights reserved. Published by The University of Chicago Press for the Society for Freshwater Science. <https://doi.org/10.1086/719359>

scenarios (Buchwalter et al. 2017). For example, the use of laboratory experiments on established model species, such as *Ceriodaphnia dubia* (Richard, 1894) (Armstead et al. 2016), have been inappropriately applied to assess the safety of total dissolved solids pollution in systems with declining mayfly populations (Pond et al. 2008). Chironomids are also widely used as model species, but species in this family are typically tolerant of many environmental stressors (Buchwalter et al. 2004, Hassell et al. 2006, Raby et al. 2018). No single species is a perfect representation of the extreme biodiversity found among freshwater organisms (Dijkstra et al. 2014), but a more ecologically defensible toxicity model species for total dissolved solid pollution in streams is needed (Sibley et al. 2020).

Because there are knowledge gaps in the toxicological responses to salinity in sensitive aquatic insects, our lab, among others, has been working to develop a lab-reared model organism, *Neocloeon triangulifer* (McDunnough, 1931). This small baetid mayfly performs well under laboratory conditions and is a parthenogenetic species with an ~25-d larval lifespan at room temperature (21–23°C; Sweeney and Vannote 1984). This model species has already proven useful to learn about major ion sensitivity (Johnson et al. 2015, Soucek and Dickinson 2015, Buchwalter et al. 2018, Jackson and Funk 2019, Orr and Buchwalter 2020), ion transport rates (Poteat and Buchwalter 2014, Scheibener et al. 2017, Orr and Buchwalter 2020, Orr et al. 2021), and gene expression patterns in response to abiotic stressors (Kim et al. 2017, Chou et al. 2020, Orr et al. 2021). However, the degree to which different larval life stages (Figs 1A–C, 2A–C) affect sensitivity and physiological endpoints, such as ion uptake rates, remains unclear.

Importantly, many aquatic insect species exhibit greater sensitivity and mortality as juveniles than as adults (Nebeker et al. 1984, Gosselin and Qian 1997, Buchwalter et al. 2004, Mebane et al. 2008). For example, the freshwater shrimp *Halocaridina rubra* (Holthuis, 1963) demonstrates molecular developmental changes in salinity tolerance and energy acquisition across life stages (Havird and Santos 2016). Few studies, however, have examined age differences in sen-

sitivity of aquatic insects, likely because of the difficulty of culturing aquatic insects in the laboratory. Some mesocosm studies have examined the impact of metals across multiple differently-aged aquatic insect taxa (Kiffney and Clements 1996, Clark and Clements 2006, Clements et al. 2013). Other studies have found increased sensitivity in early mayfly instars to orthophosphate and fine sediment (*Serratella ignita* [Poda, 1761]; Everall et al. 2018) and zinc (*Baetis tricaudatus* [Dodds, 1923]; Cadmus et al. 2020).

In this study, we aimed to assess the sensitivity of *N. triangulifer* to salinity at different stages of development. We hypothesized that *N. triangulifer* larvae would be more sensitive at early-stage development because of previous findings in other aquatic taxa. Because earlier work demonstrates a direct relationship between ion flux rates and toxicity (Orr and Buchwalter 2020), we also investigated differences of ion uptake rates between larvae of different age groups. We hypothesized that younger larvae may have increased ion flux because of their small surface area to volume ratio. Finally, we discuss the need to establish and standardize an appropriate mayfly model to protect freshwater ecosystems from salinization.

METHODS

To assess differences in the sensitivity of *N. triangulifer* to salinization at different stages of development, we performed a series of 96-h toxicity tests with 3 different salts (NaCl, CaCl₂, and MgSO₄/CaSO₄) across multiple larval stages to reflect the predominate total dissolved solids issues in different systems. Then, we recorded survival after 96-h for each test ($n = 3–6$), performed a general linear model analysis to assess effects of life stage, ion treatment, and ion concentration on survival, and calculated median lethal concentrations (LC₅₀) values for each salt and larval stage. For SO₄ results, we used previous chronic data to calculate acute-to-chronic ratios (ACR), which represent the relationship between modes of action of acute and chronic toxicity. Additionally, we characterized ion uptake rates in time course experiments using radiotracers for Na, Ca, and SO₄



Figure 1. Larval stages of *Neocloeon triangulifer* (McDunnough, 1931). One-d-old hatchling (A), ~20-d-old larvae (B), and ~25-d-old larvae with dark wing pads (white arrow) (C). Photo credit: SEO.

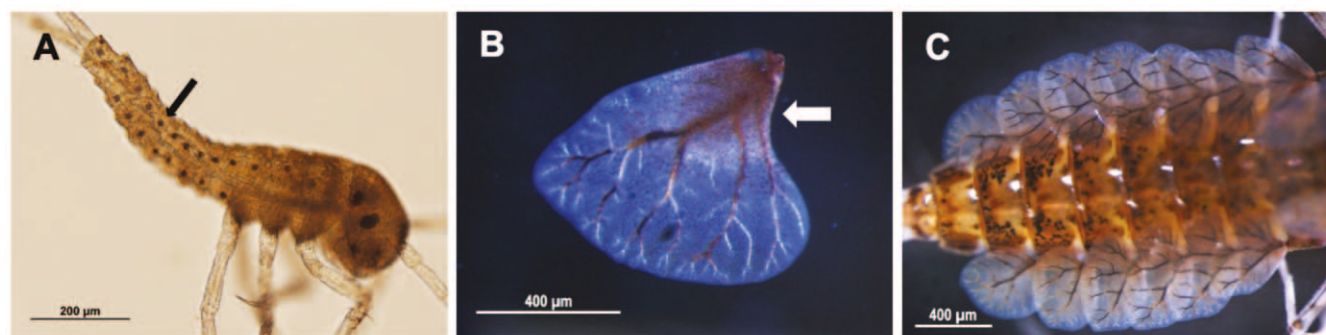


Figure 2. Ionocyte staining of *Neocloeon triangulifer* (McDunnough, 1931) larvae. One-d-old larva without gills with several large ionocytes along the thorax (black arrow) and abdomen (A); a single gill plucked off a mature (~25 d old) larva, revealing hundreds of ionocytes concentrated at the medial part of the gill (white arrow) (B); and the abdomen of a mature larvae with 14 large gills (C). Photo credit: SEO.

in 2 larval stages to help explain our toxicity observations ($n = 6$; 12, 18, 24 h for Ca and 3, 6, 9 h for Na and SO_4). Then, we conducted a student's t -test to assess differences between calculated uptake rates.

Mayfly culture

We reared mayflies in the laboratory to obtain populations at different stages of larval development based on age. *Neocloeon triangulifer* larvae were originally obtained from the White Clay Creek in Pennsylvania, USA, (Clone WCC-2) by our collaborators at the Stroud Water Research Center in Avondale, Pennsylvania (Sweeney and Vannote 1984). We reared mayfly larvae in room temperature (21–23°C) control water (artificial soft water [ASW]) or very soft water [VSW]; Table 1), which were made with recipes obtained from the United States Environmental Protection Agency (D. Mount, Environmental Protection Agency, Duluth, Minnesota, 2017, personal communication). All water was made using pure distilled water (18.0 megohm) with laboratory-grade salts (Thermo Fisher Scientific, Waltham, Massachusetts). Rearing took place on the bench top in 200-mL glass Pyrex® dishes (South Greencastle, Pennsylvania) with a 14:10-h light:dark photoperiod. We lightly aerated rearing dishes to provide highly oxygenated water and covered them in parafilm to prevent evaporation. We fed mayflies natural periphyton ad libitum on acrylic plates (6.5 × 23 × 0.15 cm).

Ionocyte staining

We stained live *N. triangulifer* larvae to visualize ionocytes. After isolating a single larva onto a glass microscope slide, we applied several drops of 2% AgNO_3 solution. After resting in direct light for ~2 m, we imaged mayflies on a MZ 16F stereoscope (Leica, Wetzlar, Germany) or BX41-P light microscope (Olympus Life Science, Center Valley, Pennsylvania). For visualization of late-stage larvae, we followed the same method but removed gills and imaged them separately.

Toxicity tests

We made experimental waters for toxicity tests with a base of ASW for Na and Ca or VSW for SO_4 . For convenience, we describe our waters by the 3 major ions (Na^+ , Ca^{2+} , and SO_4^{2-}), but we acknowledge that the companion ions associated with these salt additions may also contribute to toxicity. Control waters contained low levels of major ions: 15 mg/L Na (ASW), 12 mg/L Ca (ASW), and 23 mg/L SO_4 (VSW) (Table 1). We amended Na and Ca waters with NaCl and CaCl_2 , respectively. We made sulfate waters with a blend of both CaSO_4 and MgSO_4 because of relatively low solubility of CaSO_4 and to reduce the likelihood of cation effects. We chose a series of concentrations for each major ion of interest based on previous environmental and laboratory assessments: NaCl: 15, 205, 280, 387, 535, or 743 mg/L Na (ASW); Ca/ MgSO_4 : 23, 378, 630, 1050, 1750, or 2500 mg/L SO_4 (VSW); CaCl_2 : 12, 127, 212, 352, 588, or 980 mg/L Ca (ASW). All waters were filtered through nylon 0.45- μm syringe filters (Thermo Fisher Scientific) into sterile 15-mL test tubes (Olympus Life Science). Several major ion concentrations were verified by North Carolina State University's Environmental and Agriculture Testing Services Lab with inductively coupled plasma mass spectrometry and were within 10% of nominal values, except 743 mg/L Na, which was 21% higher than expected (Table 1). Because of the expensive nature of this technique, only some of the relevant major ions were measured. We used an Orion 5-Star™ Benchtop pH meter (Thermo Fisher Scientific) to measure the pH of all waters, which were in the expected range of 6.91 to 7.89 (Table 1).

To assess survival across different concentrations of major ions, we performed acute 96-h toxicity tests in sterile 6-well plates. The toxicity test experiments were conducted with different ages of *N. triangulifer* (2–6, 9–13, 17–21, and 23–27 d old, with the 23–27 d group for CaCl_2 experiments only). Four larval life stages were tested for CaCl_2 but not other salts of interest because of the number of available animals. Our toxicity test protocol has been previously

Table 1. Water chemistries (mg/L) for all experimental solutions. All waters had several ions verified by inductively coupled plasma mass spectrometry. AWS = artificial soft water, VSW = very soft water, Na = sodium, Ca = calcium, Mg = magnesium, K = potassium, S = sulfur, SO₄ = sulfate, Cl = chloride, CO₃ = carbon trioxide, – = no data.

Solution	Na		Ca		Mg		K		S		SO ₄	Cl	CO ₃	pH
	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured				
Control (ASW)	15.3	15.5	12.7	11.6	3.4	3.3	1.4	1.9	7.78	7.9	23.3	14.1	42.6	7.0
Control (VSW)	15.3	15.3	4.1	4.68	0.9	0.9	1.4	–	1.8	1.9	5.4	4.6	12.3	7.8
127 Ca	15.3	15.3	127	124	3.4	3.3	1.4	–	7.78	7.9	23.3	201	42.6	7.3
212 Ca	15.3	14.7	212	203	3.4	3.8	1.4	–	7.78	7.1	23.3	335	42.6	7.3
352 Ca	15.3	15.1	352	330	3.4	3.3	1.4	–	7.78	7.5	23.3	559	42.6	7.1
588 Ca	15.3	15.5	588	546	3.4	3.6	1.4	–	7.78	7.8	23.3	933	42.6	7.0
980 Ca	15.3	15.5	980	915	3.4	3.8	1.4	–	7.78	7.9	23.3	1555	42.6	6.9
205 Na	205	228	12.7	11.56	3.4	–	1.4	–	7.78	7.9	23.3	334	42.6	7.7
280 Na	280	298	12.7	12.26	3.4	–	1.4	–	7.78	8.7	23.3	452	42.6	7.8
387 Na	387	414	12.7	11.69	3.4	–	1.4	–	7.78	8.2	23.3	615	42.6	7.6
535 Na	535	559	12.7	11.83	3.4	–	1.4	–	7.78	8.7	23.3	844	42.6	7.8
743 Na	743	915	12.7	11.99	3.4	–	1.4	–	7.78	8.6	23.3	1165	42.6	7.9
378 SO ₄	15.3	16.6	53.2	52.6	63.9	60.9	1.4	–	126	128	378	4.6	12.3	7.5
630 SO ₄	15.3	16.8	88.7	85.1	107	103.0	1.4	–	210	212	630	4.6	12.3	7.4
1050 SO ₄	15.3	17.0	148	143.6	178	178.1	1.4	–	351	365	1050	4.6	12.3	7.4
1750 SO ₄	15.3	17.4	246	232.5	296	291.4	1.4	–	585	601	1750	4.6	12.3	7.3
2500 SO ₄	15.3	17.1	351	327.0	423	414.5	1.4	–	836	862	2500	4.6	12.3	7.3

described in detail (Orr and Buchwalter 2020, Orr et al. 2021), but here we give a brief summary. We filled wells ~75% full (8 mL) with experimental solution. For each age group, we seeded 10 *N. triangulifer* larvae into each well ($n = 3$ wells/treatment) with a glass pipette and the Leica MZ 16 F stereoscope. Then, we added 200 μ L of diatom slurry prepared in each exposure concentration as food. This procedure maintained the desired salinity treatment regimes. We kept the plates spatially randomized in an incubator at 21°C. We monitored and aerated the plates (60 s/well) daily. We performed a 50% water change after 48 h to ensure no excess debris and waste accumulated. We measured survivorship in each well after 96 h.

We used a general linear model (Prism, version 9.0.1; GraphPad Software, La Jolla, California) to assess how strongly survivorship varied with ion type, ion concentration, and age group (2–6, 9–13, or 17–21 d). Survivorship data were arcsine transformed prior to analysis. Ion concentrations were expressed in mM. We verified normality of model residuals based on visual examination of a Q-Q plot and a Shapiro–Wilk test ($p = 0.10$). The model was first fit with all possible interaction terms as: survival = $f(\text{ion} + \text{concentration} + \text{age} + \text{conc:age} + \text{conc:ion} + \text{age:ion} + \text{age:conc:ion})$. Then, because the parameter estimates for all interaction terms had $p > 0.05$, we dropped them from the model. Because our data were unbalanced, we intentionally chose Type II sums of squares, which is appropriate for models without interactions and only main effects (Hector et al. 2010).

We calculated LC_{50} values as concentrations of Na^+ for NaCl tests or SO_4^{2-} for Ca/Mg SO_4 tests and estimated 95% confidence intervals for each ion treatment with the Toxicity Relationship Analysis Program (version 1.30a; United States Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, Minnesota), which incorporates classic probit analysis ($n = 3$ –6 for each treatment within each larval age group) (Erickson 2010). In the Ca toxicity experiment, we were unable to calculate LC_{50} values because of high survivorship.

In addition, we calculated the approximate ACR for SO_4 based on previous chronic SO_4 data and our LC_{50} values. First, we used previous chronic data in *N. triangulifer* (Buchwalter et al. 2018) to calculate the chronic value (ChV) by taking the geometric mean of the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC). Then, using our LC_{50} data for SO_4 , we calculated ACR using the equation $ACR = LC_{50}/ChV$ for each larval life stage. These ACR values are only approximate because the LC_{50} and ChV values used to calculate the ACR values were determined in separate, independent tests instead of in the more traditional concurrent, paired acute and chronic toxicity tests.

Ion flux experiments

To assess the effect of larval stage on ion flux rates, we conducted ion flux experiments on 2 ages of mayfly larvae.

We reared mayfly hatchlings from the same cohort in control water until they reached 18 or 25 d old. We chose these age groups based on our ability to experimentally work with the smallest (18 d old) and largest (25 d old) size possible. We used the Leica MZ 16 F stereoscope with Leica camera and Leica Application Suite X software (version 4.13) to measure lengths of *N. triangulifer* larvae. We made radioactive waters in ASW with $^{45}CaCl_2$ or dual-labeled with $^{22}NaCl$ and $Na_2^{35}SO_4$ (PerkinElmer®, Billerica, Massachusetts) with exposure activities ranging from 156 to 260 Bq/mL. We measured experimental waters with an LS6500 multipurpose scintillation counter (Beckman Coulter, Brea, California). Experiments were performed in clean, acid-washed, 100-mL high-density polyethylene beakers with 20 mL of experimental water that were gently aerated and sealed with ParaFilm™ M Wrapping Film (Thermo Fisher Scientific) to prevent evaporation. Each experiment had 3 mayflies in each of 6 replicate chambers spatially randomized for each of the 3 time points (12, 18, 24 h for Ca and 3, 6, 9 h for Na and SO_4). Relatively short time points are required to capture unidirectional uptake rates, as described previously (Orr and Buchwalter 2020). Longer time points for Ca were required because Ca uptake is physiologically much slower than Na or SO_4 .

At the designated experimental time points, we removed mayflies from the radioactive water and prepared them for radioactivity analysis. First, we rinsed removed mayflies in 2 consecutive baths of clean water to remove any adsorbed ions from exoskeletons. For the Ca experiments, mayflies were additionally rinsed with freshly made 0.05 M ethylenediaminetetraacetic acid and 0.1 M L-ascorbic acid Na salt to remove adsorbed Ca on the exoskeleton (Poteat and Buchwalter 2014). We then blotted mayflies dry with a tissue, weighed them, and digested them in a 20-mL glass vial with 500 μ L of Soluene® 350 (PerkinElmer) for 48 h in a dark, 28°C incubator. After digestion, we neutralized samples with 500 μ L of glacial acetic acid. To quantify radioactivity, we added 12 mL of scintillation cocktail (PerkinElmer Ultima Gold™ uLLT) before measuring radioactivity of samples (counts/min) for 3 min each with the Beckman LS6500 multipurpose scintillation counter. We corrected all measurements for quench, which is the interference of sample and cocktail characteristics on the radioactivity quantification. Only measurements with counting error values <10% and lumex (non-radioactive luminescence) values <5% were included for analysis, which has historically been our lab's conservative data quality threshold for quantifying radioactivity.

We calculated ion uptake rates by normalizing the amount of radiolabeled ion uptake to the wet mass of tissue to account for differences in mass among the replicates. Next, we performed a simple linear regression of mass-normalized ion uptake on time in the Prism software and used the positive slope of the regression as the ion uptake rate. We assessed all models for normality of residuals through visual examination of Q-Q plots. Then, we used Student's *t*-tests to determine

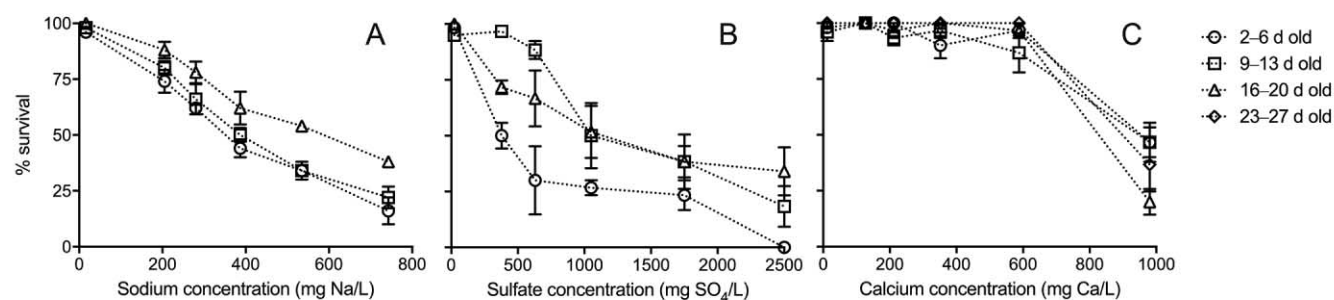


Figure 3. % survival of different ages of *Neocloeon triangulifer* (McDunnough, 1931) larvae after 96 h of exposure to sodium chloride (A), sulfate (a mixture of calcium sulfate and magnesium sulfate) (B), or calcium dichloride (C). Error bars represent SE of the mean.

if uptake rates varied between larval stage (18 or 25 d) for each ion.

RESULTS

Toxicity tests

Mayfly survival generally decreased with increasing major ion concentration for all 3 salts of interest (Fig. 3A–C). For NaCl, life stages had LC₅₀ values of 401 mg Na/L (2–6 d), 442 mg Na/L (9–13 d), and 570 mg Na/L (17–21 d) (Table S1, Fig. 4A). Mayfly survival decreased at the highest Ca concentration across all 4 larval life stages (Table S3, Fig. 3C). Because of high survival, we were unable to calculate LC₅₀ values. Mayfly survival also generally decreased with increasing SO₄ concentration (Table S2, Fig. 3B). The 3 different larval life stages had LC₅₀ values of 749 mg SO₄/L (2–6 d), 1503 mg SO₄/L (9–13 d), and 1439 mg SO₄/L (17–21 d) (Table S2, Fig. 4B). Based on previous chronic SO₄ work in *N. triangulifer*, we were able to use the NOEC (444 mg SO₄/L) and the LOEC (667 mg SO₄/L) to calculate

the ChV (544 mg SO₄/L) based on mean days to emergence (Buchwalter et al. 2018). Then, using the current LC₅₀ data from each of the 3 life stages, we calculated approximate ACR (LC₅₀/ChV) as 1.4, 2.8, and 2.6, respectively.

Based on the general linear model results (Table 2, model $R^2 = 0.71$), all 3 variables appeared to influence mayfly survival (ion: $p < 0.0001$; age: $p = 0.0065$; concentration: $p < 0.0001$). However, the effects of ion and concentration were much stronger than the effects of age.

Ion flux experiments

Neocloeon triangulifer larvae had similar ion uptake rates at 18 and 25 d old. Larvae were 1.57 ± 0.04 and 3.26 ± 0.12 mg wet mass ($n = 54$, $p < 0.0001$) at 18 and 25 d old, respectively. For 18- and 25-d-old larvae, Na uptake rates were 49.1 ± 5.8 and $39.3 \pm 3.2 \mu\text{g g}^{-1} \text{h}^{-1}$, SO₄ uptake rates were 5.7 ± 3.0 and $8.1 \pm 1.8 \mu\text{g g}^{-1} \text{h}^{-1}$, and Ca uptake rates were 5.3 ± 0.1 and $4.5 \pm 0.6 \mu\text{g g}^{-1} \text{h}^{-1}$, respectively (Fig. 5). Student's *t*-tests revealed that larval stage did not substantially affect

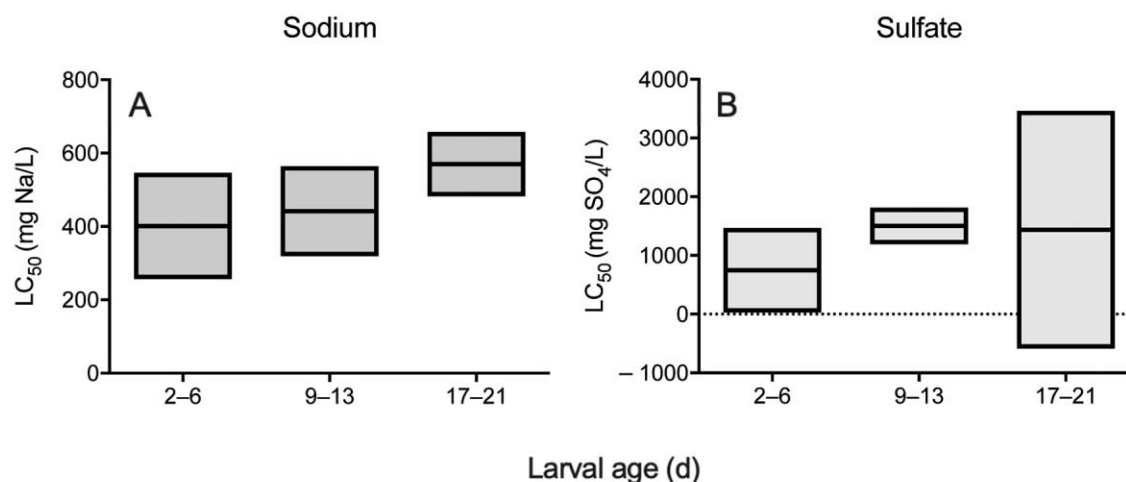


Figure 4. Calculated median lethal concentrations (LC₅₀ values) for 3 different ages of *Neocloeon triangulifer* (McDunnough, 1931) larvae exposed to different NaCl (A) or Ca/MgSO₄ (B) concentrations. Error bars represent 95% confidence limits.

Table 2. General linear model analysis of the effects of ion type, larvae age, and ion concentration on survivorship of *Neocloeon triangulifer* (McDunnough, 1931) larvae. SS = sum of squares, MS = mean sum of squares, – = no data.

Analysis of variance	SS	df	MS	F	p
Regression	47.27	5	9.454	111.4	<0.0001
Ion	7.67	2	3.833	45.18	<0.0001
Age	0.87	2	0.437	5.15	0.0065
Concentration	34.08	1	34.08	401.6	<0.0001
Residual	19.60	231	0.084	–	–
Total	66.87	236	–	–	–

ion uptake rates for Na ($p = 0.17$), SO_4 ($p = 0.53$), or Ca ($p = 0.22$).

DISCUSSION

In this study, we aimed to uncover differences of major ion sensitivity between larval stages of the mayfly *N. triangulifer*. We found that for all 3 salts (NaCl, Ca/MgSO₄, and CaCl₂) older larvae were slightly more tolerant than younger larvae. Further, we found weak differences in ion transport rates of Na, SO_4 , and Ca in 2 differently-aged groups of larvae. These results have implications for establishing water-quality criteria for salinity in freshwater ecosystems and point to the need to establish a model organism (*N. triangulifer*) to improve our understanding of aquatic insect osmoregulation and sensitivity.

Freshwater salinization

Freshwater salinization is an emerging issue that merits scientific attention and regulatory action to mitigate ongoing harmful ecological impacts (Pond et al. 2008, Stepanian et al. 2020). Previous work has demonstrated the importance of assessing ion-specific effects on physiology and sensitivity rather than effects of total salinity. For example, one study found that waters with similar conductivities but different major ion compositions had different toxicities on aquatic organisms (Kunz et al. 2013). Another study showed that major ions had different mechanisms of toxicity in *N. triangulifer* (Orr et al. 2021). This information is environmentally relevant because different scenarios increase the concentration of different major ions. For example, we would expect increased Na, Mg, and Cl ions in freshwaters that are polluted by road deicing in urban areas with colder climates. Alternatively, mountaintop coal mining operations in West Virginia, USA, often cause increases in SO_4 and Ca ions in nearby freshwater systems (Pond et al. 2008, Cormier et al. 2013, Jackson and Funk 2019).

Aquatic insects

Aquatic insects are disproportionately affected by increases in major ion concentrations (Pond et al. 2008, Grif-

fith 2017) compared with other aquatic organisms, but their sensitivity is not well understood. Chemical benchmarks for altered major ion concentrations have been developed from field surveys of aquatic invertebrate communities (Cormier and Suter 2013a, b) but are not yet legally enforceable. Because these approaches cannot determine mechanisms of toxicity nor identify which part of the life cycles of sensitive aquatic insects are affected, it is important that we develop a better understanding of how total dissolved solids affect aquatic organisms. Sensitive aquatic organisms, such as mayflies, are challenging to study because of their intolerance of laboratory conditions. However, the development of *N. triangulifer* as a lab-reared model organism has opened many doors to ecotoxicology research in sensitive aquatic insects (Sweeney and Vannote 1984, Sweeney et al. 1993). Scientists can rear this species through its entire life cycle in laboratory conditions and produce valuable chronic toxicity data for salts and other stressors (Soucek and Dickinson 2015, Buchwalter et al. 2018, Jackson and Funk 2019, Chou et al. 2020). Here, we used this developing model organism, *N. triangulifer*, and produced valuable toxicity data for 3 different water chemistries. Our results are an important contribution to the growing body of data on salinity stress in aquatic life, taking into consideration both larval life stage and different major ions.

Acute and chronic toxicity to major ions

We examined 3 different salts of interest because previous findings have demonstrated clear physiological and toxicological differences between major ions (Kunz et al. 2013, Scheibener et al. 2017, Orr and Buchwalter 2020, Orr et al. 2021). Larvae tolerated Cl^- concentrations as high as 933 mg/L in the CaCl₂ treatments, which leads us to speculate that Na is driving NaCl toxicity. We presume that Ca is

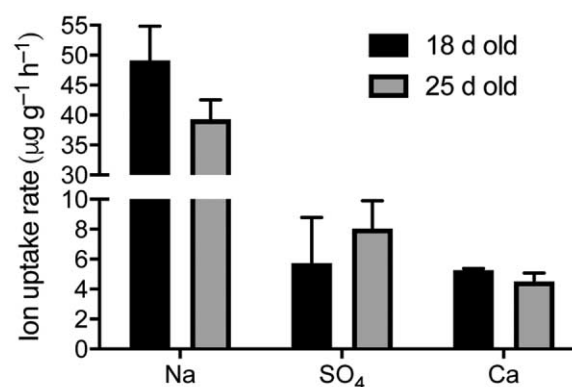


Figure 5. Ion uptake rates in artificial soft water of *Neocloeon triangulifer* (McDunnough, 1931) larvae at 18 d and 25 d old ($n = 6$; $p = 0.17$, $p = 0.53$, and $p = 0.22$ for sodium [Na], sulfate [SO_4], and calcium [Ca], respectively). Error bars represent the SE of the mean. Note the break and change in y-axis scale.

the driver of CaCl_2 toxicity because the larvae tend to maintain a very low uptake rate of Ca, and we have observed histological changes in Malpighian tubules (Orr et al. 2021). Our concentration choices were made based on previous laboratory results and environmental values, and our acute toxicity data is relatively consistent with the literature. Recently, our group calculated LC_{50} values for Na and Ca with ~10- to 17-d-old *N. triangulifer* larvae in similar water chemistries: 1169 mg Na/L and 901 mg Ca/L, respectively (Orr et al. 2021). Other groups have calculated LC_{50} values for SO_4 (1227 mg/L; Soucek and Dickinson 2015) and NaCl (2755 mg/L, equivalent to 1084 mg N/L; Jackson and Funk 2019). Some potential explanations for differing results between studies include different natural periphyton quality based on seasons, different cohorts, or a combination of both. Our results emphasize the importance of studying salts of major ions individually rather than using total salinity or conductivity as a surrogate in salinity research. It is important to note, however, that animals are exposed to several major ions (among other stressors) in nature and that further work should be done with different combinations of relevant salts to improve our understanding of ionic interactions.

Historically, the United States has relied upon ACRs in ecological risk assessment to estimate chronic toxicity of chemicals for aquatic species that lack chronic data (Stephan et al. 1985, Raimondo et al. 2007). Normally, ACRs are calculated as a ratio of the median LC_{50} and the ChV, which can only be done for species that have both acute and chronic data (Raimondo et al. 2007). Thus, a larger ACR value (>10) would indicate different modes of action between acute and chronic exposures. Here, our calculated approximate ACR values were low, suggesting major ions elicit similar mechanisms of toxicity for both acute and chronic exposures in this species. Using similar endpoints, one study found ACRs ranging from 2.3 to 8.5 for SO_4 in this species (Soucek and Dickinson 2015). The differences may be explained by variances in nutritional provisions, ionic compositions of the water, or a combination of both. Soucek and Dickinson (2015) used laboratory-cultured diatoms and NaSO_4 rather than natural periphyton and a blend of CaSO_4 and MgSO_4 . At least some of the variability in the ACR values might also be a result of determining the LC_{50} values in a separate, independent study from that used to determine the ChV values. Soucek and Dickinson (2015) also calculated ACRs for Cl^- (2.1–6.4) and NO_3^- (2.5–5.1) in *N. triangulifer*, using NaCl and NaNO_3 , respectively (Soucek and Dickinson 2015). Additional chronic toxicity tests are needed to fully characterize the toxicity of the salts of different major ions and then develop predictive toxicity models.

Ion uptake rates

Previous work has demonstrated the highly concentration-dependent and ion-specific nature of ion uptake rates and, further, that increased ion flux rates are associated with in-

creased toxicity (Orr and Buchwalter 2020, Orr et al. 2021). We hypothesized that smaller animals may have greater ion flux rates because of their increased surface area-to-volume ratio. Here, we observed that basal ion uptake rates in control water remain unchanged between 18- vs 25-d-old larvae from the same cohort. This result aligns with the marginal differences in LC_{50} values observed between differently-aged larvae in this study.

Life-stage sensitivity

Aquatic insects develop through various life stages and may be more sensitive to metals and other toxicants during transitional stages that face steep energetic demands. Research on other aquatic insects has demonstrated greater sensitivity to toxicants in the transitional stages, including the last molting stage and metamorphosis (Palmquist et al. 2008, Schmidt et al. 2013, Wesner et al. 2017, Wesner 2019). Similarly, we have often observed mortality in large, late-stage *N. triangulifer* larvae soon before emergence in chronic salinity experiments (SEO, DBB, personal observations, unpublished), which may suggest that energy budget, rather than size, determines sensitivity. A previous study demonstrated that pre-exposure of *N. triangulifer* to elevated, but subtoxic, SO_4 concentrations stimulated an acclimatory response (reduction in SO_4 uptake rates) but also increased subsequent toxicity (Orr et al. 2021). Molting drastically disrupts breathing and metabolic rate in this species (Camp et al. 2014), and greater mortality has been observed at the penultimate larval stage of a chronic NaCl exposure, emphasizing the energetic demand of metamorphosis combined with stressful salinities (Soucek and Dickinson 2015). Future studies should focus on fully understanding the energy requirements and increased sensitivity of the transition from the penultimate larva to subimago in *N. triangulifer*.

Neocloeon triangulifer as a model species

Nutrition is an important consideration when using *N. triangulifer* as a model species. Previous work has shown that nutrition can alter selenium toxicity in this species (Conley et al. 2011). Many groups have reared *N. triangulifer* on natural periphyton (Jackson and Funk 2019, Orr and Buchwalter 2020, Orr et al. 2021), whereas others have used laboratory-grown diatoms (Weaver et al. 2014, Soucek and Dickinson 2015, Raby et al. 2018). A more heterogeneous, natural mixture of periphyton may provide better nutrition but is impossible to standardize. On the other hand, cultured diatoms may not provide optimal nutrition but may better reflect ecological realities in some settings. Because we posit that high total dissolved solids impose energetic costs to developing larvae, we recommend that researchers using *N. triangulifer* report the mass of suitably sized controls when possible so that results can be interpreted in the context of nutritional status.

Further, this species is relatively small compared with other species of mayflies and aquatic insects (Jackson and Funk 2019), which affects their use in toxicity tests. Morphologically, these animals undergo major changes from hatchlings with no gills and few ionocytes to the penultimate larval stage with 14 large gills and thousands of ionocytes concentrated on the medial part of the gills (Fig. 2A–C). Thus, it appears that the number of cells is commensurate with size of the larvae. Notably, most stages of this species' larval life cycle can only be worked with experimentally using a microscope. For these reasons, we often use mature larvae close to the end of their larval cycle for ease of use and to maximize biomass, which is important in radiotracer studies that use a minimal amount of radioactivity for safety concerns (Scheibener et al. 2017, Orr and Buchwalter 2020), among other experiments. It is clear from our results that mature larvae are slightly more tolerant of major ions and, thus, experimental larval stage should be carefully considered in future studies. However, it appears that the egg stage of *N. triangulifer* is relatively intolerant to salinity stress (D. Funk, Stroud Water Research Center, Avondale, Pennsylvania, 2021, personal communication). We speculate that early larval life stages in aquatic organisms may be more sensitive because of greater surface area-to-volume ratios, faster turnover rate of essential ions, and underdeveloped antioxidant and immune systems.

In this study, we tested for sensitivity differences between larval life stages of *N. triangulifer* in a laboratory setting, but we acknowledge that acute toxicity tests, conducted in the absence of other biotic and abiotic stressors, are not a reasonable proxy for insect sensitivity in nature (Kefford et al. 2004, Hassell et al. 2006, Vellemu et al. 2017). However, lab-based approaches allow us to perform controlled experiments to isolate the effects of different major ions and broaden our understanding of how they affect survivorship and other physiological endpoints. We found apparent trends of slightly increased major ion sensitivity in younger larvae. We also found weak differences in Na, SO₄, or Ca uptake rates between 2 larval stages of *N. triangulifer*. Additional in-situ stressors (e.g., temperature, weathering patterns, predation, food limitation) may exacerbate major ion toxicity. In the future, experimental studies will work in conjunction with field studies to understand the individual effects of ions on aquatic insects and the combined effects of salinization with interacting environmental stressors. Moving forward, it is clear that larval stage should be an important consideration when planning experiments studying the physiological effects and toxicity of salinity with *N. triangulifer*.

ACKNOWLEDGEMENTS

Author contributions: SEO oversaw and helped conduct the research, analyzed the data, and wrote the manuscript. JKC, IGW, RWG, and GEO helped conduct the research. DBB conceived the work and provided editorial assistance.

The research was supported by a grant to DBB (NSF-IOS 1754884), and SEO was supported by National Institute of Environmental Health Sciences Training Grant (T32ES007046). We are grateful for the periphyton plates (mayfly food) gifted to us by Stroud Water Research Center (Avondale, Pennsylvania, USA). We would also like to thank Drs Gerald LeBlanc and Chuck Hawkins for their editorial assistance.

The authors declare that they have no known competing financial or personal relationships that may have influenced the work in this manuscript.

LITERATURE CITED

- Armstead, M. Y., L. Bitzer-Creathers, and M. Wilson. 2016. The effects of elevated specific conductivity on the chronic toxicity of mining influenced streams using *Ceriodaphnia dubia*. *PLoS ONE* 11:e0165683.
- Barlow, P. M., and E. G. Reichard. 2010. Saltwater intrusion in coastal regions of North America. *Hydrogeology Journal* 18:247–260.
- Buchwalter, D., S. Scheibener, H. Chou, D. Soucek, and J. Elphick. 2018. Are sulfate effects in the mayfly *Neocloeon triangulifer* driven by the cost of ion regulation? *Philosophical Transactions of the Royal Society B: Biological Sciences* 374:0013.
- Buchwalter, D. B., W. H. Clements, and S. N. Luoma. 2017. Modernizing water quality criteria in the United States: A need to expand the definition of acceptable data. *Environmental Toxicology and Chemistry* 36:285–291.
- Buchwalter, D. B., J. F. Sandahl, J. J. Jenkins, and L. R. Curtis. 2004. Roles of uptake, biotransformation, and target site sensitivity in determining the differential toxicity of chlorpyrifos to second to fourth instar *Chironomus riparius* (Meigen). *Aquatic Toxicology* 66:149–157.
- Cadmus, P., C. J. Kotalik, A. L. Jefferson, S. H. Wheeler, A. E. McMahon, and W. H. Clements. 2020. Size-dependent sensitivity of aquatic insects to metals. *Environmental Science & Technology* 54:955–964.
- Camp, A. A., D. H. Funk, and D. B. Buchwalter. 2014. A stressful shortness of breath: Molting disrupts breathing in the mayfly *Cloeon dipterum*. *Freshwater Science* 33:695–699.
- Canedo-Arguelles, M., C. P. Hawkins, B. J. Kefford, R. B. Schafer, B. J. Dyack, S. Brucet, D. Buchwalter, J. Dunlop, O. Fror, J. Lazorchak, E. Coring, H. R. Fernandez, W. Goodfellow, A. L. González Achem, S. Hatfield-Dodds, B. K. Karimov, P. Mensah, J. R. Olson, C. Piscart, N. Prat, S. Ponsá, C.-J. Schulz, and A. J. Timpano. 2016. Saving freshwater from salts. *Science* 351:914–916.
- Chou, H., D. D. Jima, D. H. Funk, J. K. Jackson, B. W. Sweeney, and D. B. Buchwalter. 2020. Transcriptomic and life history responses of the mayfly *Neocloeon triangulifer* to chronic diel thermal challenge. *Scientific Reports* 10:19119.
- Clark, J. L., and W. H. Clements. 2006. The use of in situ and stream microcosm experiments to assess population- and community-level responses to metals. *Environmental Toxicology and Chemistry* 25:2306–2312.
- Clements, W. H., P. Cadmus, and S. F. Brinkman. 2013. Responses of aquatic insects to Cu and Zn in stream microcosms: Understanding differences between single species tests and field responses. *Environmental Science & Technology* 47:7506–7513.
- Conley, J. M., D. H. Funk, N. J. Cariello, and D. B. Buchwalter. 2011. Food rationing affects dietary selenium bioaccumulation and

- life cycle performance in the mayfly *Centroptilum triangulifer*. *Ecotoxicology* 20:1840–1851.
- Cormier, S. M., and G. W. Suter. 2013a. A method for assessing causation of field exposure-response relationships. *Environmental Toxicology and Chemistry* 32:272–276.
- Cormier, S. M., and G. W. Suter. 2013b. A method for deriving water-quality benchmarks using field data. *Environmental Toxicology and Chemistry* 32:255–262.
- Cormier, S. M., S. P. Wilkes, and L. Zheng. 2013. Relationship of land use and elevated ionic strength in Appalachian watersheds. *Environmental Toxicology and Chemistry* 32:296–303.
- Dijkstra, K.-D. B., M. T. Monaghan, and S. U. Pauls. 2014. Freshwater biodiversity and aquatic insect diversification. *Annual Review of Entomology* 59:143–163.
- Entrekin, S., M. Evans-White, and E. Hagenbuch. 2011. Rapid expansion of natural gas development poses a threat to surface waters. *Frontiers in Ecology and the Environment* 9:503–511.
- Erickson, R. J. 2010. Toxicity Relationship Analysis Program (TRAP) version 1.21. EPA/600/C-11/002. United States Environmental Protection Agency, Washington, DC. (Available from: https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NHEERL&count=10000&dirEntryId=231579&searchall=&showcriteria=2&simplesearch=0&timstype=)
- Everall, N. C., M. F. Johnson, P. Wood, and L. Mattingley. 2018. Sensitivity of the early life stages of a mayfly to fine sediment and orthophosphate levels. *Environmental Pollution* 237:792–802.
- Gosselin, L., and P. Qian. 1997. Juvenile mortality in benthic marine invertebrates. *Marine Ecology Progress Series* 146:265–282.
- Griffith, M. B. 2017. Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: Teleost fish, crustacea, aquatic insects, and Mollusca. *Environmental Toxicology and Chemistry* 36:576–600.
- Hassell, K. L., B. J. Kefford, and D. Nuggeoda. 2006. Sub-lethal and chronic salinity tolerances of three freshwater insects: *Cloeon* sp and *Centroptilum* sp (Ephemeroptera:Baetidae) and *Chironomus* sp (Diptera:Chironomidae). *Journal of Experimental Biology* 209:4024–4032.
- Havird, J. C., and S. R. Santos. 2016. Developmental transcriptomics of the Hawaiian Anchialine Shrimp *Halocaridina rubra* Holthuis, 1963 (Crustacea:Atyidae). *Integrative & Comparative Biology* 56:1170–1182.
- Hector, A., S. Von Felten, and B. Schmid. 2010. Analysis of variance with unbalanced data: An update for ecology and evolution. *Journal of Animal Ecology* 79:308–316.
- Jackson, J. K., and D. H. Funk. 2019. Temperature affects acute mayfly responses to elevated salinity: Implications for toxicity of road de-icing salts. *Philosophical Transactions of the Royal Society B: Biological Sciences* 374:0081.
- Johnson, B. R., P. C. Weaver, C. T. Nietch, J. M. Lazorchak, K. A. Struewing, and D. H. Funk. 2015. Elevated major ion concentrations inhibit larval mayfly growth and development. *Environmental Toxicology and Chemistry* 34:167–172.
- Kaushal, S. S., G. E. Likens, M. L. Pace, R. M. Utz, S. Haq, J. Gorman, and M. Grese. 2018. Freshwater salinization syndrome on a continental scale. *Proceedings of the National Academy of Sciences* 115:E574–E583.
- Kefford, B. J., P. J. Papas, L. Metzeling, and D. Nuggeoda. 2004. Do laboratory salinity tolerances of freshwater animals correspond with their field salinity? *Environmental Pollution* 129:355–362.
- Kefford, B. J., D. Buchwalter, M. Canedo-Arguelles, J. Davis, R. P. Duncan, A. Hoffmann, and R. Thompson. 2016. Salinized rivers: Degraded systems or new habitats for salt-tolerant faunas? *Biology Letters* 12:1072.
- Kiffney, P. M., and W. H. Clements. 1996. Size-dependent response of macroinvertebrates to metals in experimental streams. *Environmental Toxicology and Chemistry* 15:1352–1356.
- Kim, K. S., H. Chou, D. H. Funk, J. K. Jackson, B. W. Sweeney, and D. B. Buchwalter. 2017. Physiological responses to short-term thermal stress in mayfly (*Neocloeon triangulifer*) larvae in relation to upper thermal limits. *Journal of Experimental Biology* 220:2598–2605.
- Kinzelbach, W., P. Bauer, T. Siegfried, and P. Brunner. 2003. Sustainable groundwater management—Problems and scientific tools. *Episodes* 26:279–284.
- Kunz, J. L., J. M. Conley, D. B. Buchwalter, T. J. Norberg-King, N. E. Kemble, N. Wang, and C. G. Ingersoll. 2013. Use of reconstituted waters to evaluate effects of elevated major ions associated with mountaintop coal mining on freshwater invertebrates. *Environmental Toxicology and Chemistry* 32:2826–2835.
- Mebane, C. A., D. P. Hennessy, and F. S. Dillon. 2008. Developing acute-to-chronic toxicity ratios for lead, cadmium, and zinc using rainbow trout, a mayfly, and a midge. *Water, Air, and Soil Pollution* 188:41–66.
- Mosley, L. M. 2017. Drought impacts on the water quality of freshwater systems: Review and integration. *Earth-Science Reviews* 140:203–214.
- Nebeker, A. V., C. Savonen, R. J. Baker, and J. K. McCrady. 1984. Effects of copper, nickel and zinc on the life cycle of the caddisfly *Clistoronia magnifica* (limnephilidae). *Environmental Toxicology and Chemistry* 3:645–649.
- Orr, S. E., and D. B. Buchwalter. 2020. It's all about the fluxes: Temperature influences ion transport and toxicity in aquatic insects. *Aquatic Toxicology* 221:105405.
- Orr, S. E., T. T. Negrão Watanabe, and D. B. Buchwalter. 2021. Physiological plasticity and acclimatory responses to salinity stress are ion-specific in the mayfly, *Neocloeon triangulifer*. *Environmental Pollution* 286:117221.
- Palmquist, K. R., P. C. Jepson, and J. J. Jenkins. 2008. Impact of aquatic insect life stage and emergence strategy on sensitivity to esfenvalerate exposure. *Environmental Toxicology and Chemistry* 27:1728–1734.
- Pond, G. J., M. E. Passmore, F. A. Borsuk, L. Reynolds, and C. J. Rose. 2008. Downstream effects of mountaintop coal mining: Comparing biological conditions using family- and genus-level macroinvertebrate bioassessment tools. *Journal of the North American Benthological Society* 27:717–737.
- Poteat, M. D., and D. B. Buchwalter. 2014. Calcium uptake in aquatic insects: Influences of phylogeny and metals (Cd and Zn). *Journal of Experimental Biology* 217:1180–1186.
- Raby, M., X. Zhao, C. Hao, D. G. Poirier, and P. K. Sibley. 2018. Chronic toxicity of 6 neonicotinoid insecticides to *Chironomus dilutus* and *Neocloeon triangulifer*: Chronic toxicity of neonicotinoids to aquatic invertebrates. *Environmental Toxicology and Chemistry* 37:2727–2739.
- Raimondo, S., B. J. Montague, and M. G. Barron. 2007. Determinants of variability in acute to chronic toxicity ratios for aquatic invertebrates and fish. *Environmental Toxicology and Chemistry* 26:2019–2023.

- Scheibener, S., J. M. Conley, and D. Buchwalter. 2017. Sulfate transport kinetics and toxicity are modulated by sodium in aquatic insects. *Aquatic Toxicology* 190:62–69.
- Schmidt, T. S., J. M. Kraus, D. M. Walters, and R. B. Wanty. 2013. Emergence flux declines disproportionately to larval density along a stream metals gradient. *Environmental Science & Technology* 47:8784–8792.
- Sibley, P., L. Lagadic, M. McCooles, T. Norberg-King, I. Roessink, D. Soucek, T. Watson-Leung, and J. Wirtz. 2020. Mayflies in ecotoxicity testing: Methodological needs and knowledge gaps. *Integrated Environmental Assessment and Management* 16: 292–293.
- Soucek, D. J., and A. Dickinson. 2015. Full-life chronic toxicity of sodium salts to the mayfly *Neocloeon triangulifer* in tests with laboratory cultured food. *Environmental Toxicology and Chemistry* 34:2126–2137.
- Stepanian, P. M., S. A. Entrekin, C. E. Wainwright, D. Mirkovic, J. L. Tank, and J. F. Kelly. 2020. Declines in an abundant aquatic insect, the burrowing mayfly, across major North American waterways. *Proceedings of the National Academy of Sciences* 117:2987–2992.
- Stephan, C. E., D. I. Mount, D. J. Hansen, J. R. Gentile, G. A. Chapman, and W. A. Brungs. 1985. Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. United States Environmental Protection Agency, Office of Research and Development, Environmental Research Laboratories, Duluth, Minnesota.
- Sweeney, B. W., D. H. Funk, and L. J. Standley. 1993. Use of the stream mayfly *Cloeon triangulifer* as a bioassay organism: Life-history response and body burden following exposure to technical chlordane. *Environmental Toxicology and Chemistry* 12:115–125.
- Sweeney, B. W., and R. L. Vannote. 1984. Influence of food quality and temperature on life-history characteristics of the parthenogenetic mayfly, *Cloeon triangulifer*. *Freshwater Biology* 14:621–630.
- USEPA (United States Environmental Protection Agency). 1988. Ambient water quality criteria for chloride. EPA 44015-88-001. United States Environmental Protection Agency, Office of Research and Development, Environmental Research Laboratories, Duluth, Minnesota.
- Vellemu, E. C., P. K. Mensah, N. J. Griffin, and O. N. Odume. 2017. Sensitivity of the mayfly *Adenophlebia auriculata* (Ephemeroptera:Leptophlebiidae) to $MgSO_4$ and Na_2SO_4 . *Physics and Chemistry of the Earth: Parts ABC* 100:81–85.
- Weaver, P. C., J. M. Lazorchak, K. A. Struewing, S. J. DeCelles, D. H. Funk, D. B. Buchwalter, and B. R. Johnson. 2014. Part 1: Laboratory culture of *Centroptilum triangulifer* (Ephemeroptera:Baetidae) using a defined diet of three diatoms. *Chemosphere* 139:589–596.
- Wesner, J. 2019. Using stage-structured food webs to assess the effects of contaminants and predators on aquatic–terrestrial linkages. *Freshwater Science* 38:928–935.
- Wesner, J. S., D. M. Walters, T. S. Schmidt, J. M. Kraus, C. A. Stricker, W. H. Clements, and R. E. Wolf. 2017. Metamorphosis affects metal concentrations and isotopic signatures in a mayfly (*Baetis tricaudatus*): Implications for the aquatic–terrestrial transfer of metals. *Environmental Science & Technology* 51: 2438–2446.