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Are sulfate effects in the mayfly *Neocloeon triangulifer* driven by the cost of ion regulation?

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Elevated major ion concentrations in streams are commonly observed as a consequence of resource extraction, de-icing and other anthropogenic activities. Ecologists report biodiversity losses associated with increasing salinity, with mayflies typically being highly responsive to increases of different major ions. In this study, we evaluated the performance of the mayfly *Neocloeon triangulifer* reared for its entire larval phase in a gradient of sulfate concentrations. Two natural waters were amended with SO_4 as a blend of $CaSO_4$ and $MgSO_4$ and exposures ranged from 5 to 1500 mg l^{-1} SO_4. Survival (per cent successful emergence to the subimago stage) was significantly reduced at the highest SO_4 concentration in both waters, while development was significantly delayed at 667 mg l^{-1} SO_4. Final sub-adult body weights were consistent across treatments, except at the highest treatment concentration. Despite evidence for sulfate uptake rates increasing with exposure concentrations and not being saturated at even extremely high SO_4 concentrations, total body sulfur changed little in subimagos. Together, these results suggest that elevated SO_4 imposes an energetic demand associated with maintaining homeostasis that is manifested primarily as reduced growth rates and associated developmental delays. We identified two genes related to sulfate transport in *N. triangulifer*.

This article is part of the theme issue 'Salt in freshwaters: causes, ecological consequences and future prospects'.

1. Introduction

Freshwater salinization has emerged as a major issue in freshwater ecosystems throughout the world [1–6]. Anthropogenic causes of salinization include road salting [7,8], irrigation of arid lands [9,10], urbanization [11,12], natural gas extraction and associated brine spills [13,14] and mountaintop removal/valley fill mining [6]. Climatic related changes in freshwater salinities include altered precipitation patterns and drought [15] and seawater intrusion from rising sea levels. These diverse causes of salinization occur in different geologies and result in a wide variety of salinity signatures. It is important that we significantly improve our understanding of how different salinity regimes affect aquatic life and how they might be mitigated.

Surface coal mining in many parts of the world can result in rather unique stream chemistries marked by dramatic increases in sulfate, bicarbonate, calcium and magnesium [6]. These streams are often low in the concentrations of other ions (e.g. Na and Cl) commonly observed to be elevated in other anthropogenic causes of increased salinity. Ecological responses to sulfate-dominated increases in salinity include the loss of mayflies and other taxa [6], which may be locally adapted to dilute habitats.

Because mayflies are important players in freshwater ecosystems and are responsive to environmental changes, several laboratories have begun working

with *Neocloeon triangulifer* (formerly *Cloeon triangulifer* and *Centroptilum triangulifer*) as a mayfly model to explore responses to elevated salinity. *Neocloeon triangulifer* is one of a very few mayfly species that has been cultured successfully in the laboratory. Sweeney and co-workers [16,17] were the first to culture this species and use it in a bioassay context. Decades later, studies by Xie and co-workers [18,19], Conley *et al.* [20–23] and Kim *et al.* [24] demonstrated the use of this species to study trace metal accumulation and trophic exposures through periphytic biofilms. Other laboratories soon followed suit [25–27], and the United States (US) Environmental Protection Agency (EPA) began work to standardize rearing conditions so that the species could be used in different laboratories with established rearing protocols [28,29].

Scheibener *et al.* [30] was the first to study sulfate transport kinetics in *N. triangulifer* and other stream insects, and demonstrated that increasing sodium (Na) concentrations resulted in reduced sulfate uptake from solution, which corresponded with reduced sulfate toxicity on an acute basis. This finding was important, because the use of sodium sulfate to asses sulfate toxicity is the most standard practice in major ion toxicity studies, e.g. [27,31]. However, Scheibener's observation suggests that the use of sodium sulfate to assess sulfate toxicity may not appropriately reflect sulfate toxicity in the low Na environments common to coal mining impacted streams.

In this study, we assessed the toxicity of sulfate in two dilute natural waters characterized by low Na (≤ 2.5 mg l^{-1}). A gradient of sulfate concentrations was prepared by spiking these natural waters with a blend of CaSO$_4$ and MgSO$_4$, maintaining a Ca : Mg mass ratio of 2.4 : 1, which was the ratio observed at site A, while site B had a ratio of 2.8 : 1. Newly hatched larvae were reared to the subimago stage across these sulfate gradients, and the percentage of larvae to successfully emergence, time to emergence and subimago weights were recorded. Total body sulfur (S) was measured in subimagos across treatments. Companion studies assessed the effect of sulfate and other major ion concentrations on mRNA expression levels using real time-quantitative polymerase chain reaction (RT-qPCR). We provide primer sequences for two genes associated with ion transport in *N. triangulifer* for the growing *N. triangulifer* research community.

2. Material and methods

(a) Test organisms and water preparation

The parthenogenetic mayfly *N. triangulifer* (clone WCC-2) was originally obtained from Stroud Water Research Center (Avondale, PA). Larvae were hatched in a modified artificial soft water (ASW) (mM: CaSO$_4 \cdot$ 2H$_2$O = 0.10, Na$_2$HCO$_3$ = 0.69, MgSO$_4 \cdot$ 7H$_2$O = 0.14, CaCl$_2$ = 0.20 and K$_2$HCO$_3$ = 0.03). This recipe was obtained from the US EPA (D. Mount 2017, personal communication) based on a meta-analysis of surface waters throughout the conterminous USA.

The base waters for the life cycle bioassay were collected from two different waterbodies at a metallurgical coal mine site in British Columbia (BC), Canada, where there was an interest in establishing a suitable limit for sulfate concentration in order to protect aquatic life. Concentrations of major ions in the waters, prior to amending with sulfate, are shown in table 1. The waters were amended with nominal concentration of 296, 444, 667, 1000 and 1500 mg l^{-1} sulfate using CaSO$_4 \cdot$ 2H$_2$O and MgSO$_4 \cdot$ 7H$_2$O (at a 2.4 : 1 mass ratio, or 1.45 molar ratio of Ca : Mg), which is consistent with the site A ratio.

Table 1. Major ion composition of the two site waters used as the base water for SO$_4$ exposures.

ion	site A (mg l^{-1})	site B (mg l^{-1})
Ca	23.0	13.9
Mg	9.3	5.0
Na	2.5	0.4
K	0.7	0.4
HCO$_3$	74.5	59.8
SO$_4$	30.1	5.6
Cl	<0.5	<0.5
TDS	119	70

Concentrations of sulfate in the test solutions at the start of the test were measured by ALS Environmental (Burnaby, BC, Canada) by ion chromatography (table 2) with a subset of samples verified by inductively coupled plasma-mass spectrometry (ICP-MS) at North Carolina State University (NCSU). At the termination of the experiment, the SO$_4$ concentrations were measured by ICP-MS in a single sample each treatment group and all were within 9% of the initial (table 2) values.

(b) Life cycle bioassay

Treatment solutions were aliquoted into replicate 1.8 l Ball^{TM} glass jars with 1.75 l of water in each jar (three replicates per treatment). In addition to stream base water controls, a modified ASW control (see above for chemistry) ($n = 4$) was also included. Newly hatched larvae were provided a diet of natural periphyton to ensure survivorship for the first 24 h post-hatch until the start of the experiment. Upon the start of the experiment, mayflies (1–2 days old) were transferred to rearing jars provisioned with acrylic plates ($6.5 \times 23 \times 0.15$ cm) colonized by an assemblage of natural periphyton (a mixture of algae, diatoms, microbiota and detritus). Periphyton plates were produced at Stroud Water Research Center by allowing stream water (White Clay Creek, PA) to flow continuously over plates contained in a greenhouse as described previously [20,21]. Plates were shipped overnight to NCSU and upon reception; one plate was placed into each replicate jar, 1 day prior to the introduction of mayfly larvae. A second plate was placed 15 days after the start of the experiment to ensure adequate food supply throughout life cycle. Water was not changed during the experiment.

Each replicate was aerated and Parafilm^{TM} was used to reduce evaporative loss, and deionized water was added to marked 'fill' lines on each vessel as needed. Conductivity, pH and dissolved O$_2$ were measured approximately 3 days per week in a minimum of two replicates per treatment. Conductivities were relatively stable for the duration of the experiment (electronic supplementary material, figure S1). pH measures ($n = 390$) averaged 8.13 ± 0.25, with the 7.57 and 8.86 being the extreme low and high measures recorded, respectively. Dissolved oxygen measures ($n = 390$) averaged 7.7 ± 0.7 mg l^{-1} through the duration of the experiment, with 5.6 mg l^{-1} being the lowest value recorded. A Hobo^{TM} temperature probe monitored temperature every hour for the entirety of the experiment and varied between 23.3 and 24.8°C. After approximately 2.5 weeks, mesh-lined collection lids were placed on top of each jar. Subimagos emerged in late afternoon/evening and were collected in 1.5 ml centrifuge tubes and placed in a −20°C freezer overnight. The mayflies were removed from the freezer, dried overnight at 60°C and weighed dry to the nearest 0.001 mg using a Sartorius CPA2P microbalance.

Table 2. Measured sulfate concentrations and total dissolved solids as sum of major ions.

nominal SO$_4$ addition (mg l^{-1})	measured SO$_4$ (mg l^{-1})		TDS (mg l^{-1})		conductivity (μS cm^{-1})	
	site A	site B	site A	site B	site A	site B
+296	330	312	525	487	729	673
+444	475	456	724	681	967	905
+667	706	678	1044	987	1307	1246
+1000	1070	1050	1540	1491	1779	1698
+1500	1640	1580	2301	2201	2386	2292

Dried subimagos were taken for whole body S concentrations across treatments from each water type. We also obtained dried subimagos from sulfate exposures conducted at the Stroud Water Research Center to assess variability in whole body S. Three individuals were composited to form a replicate and were microwave-digested (CEM MARSXpress) in 1 ml Omnitrace Ultra High Purity Nitric Acid (EMD Chemicals, Darmstadt, Germany). Filtered water samples (14 ml) were taken for each treatment pre- and post-test. Samples were filtered through 0.45 μm nylon syringe filters. Each sample was then acidified with 1 ml of Omnitrace Ultra High Purity Nitric Acid (EMD Chemicals, Darmstadt, Germany). Subimago digests and water samples were processed by the Environmental and Agricultural Testing Services Laboratory (Department of Soil Science, North Carolina State University, Raleigh, NC, USA) for quantification of major ions (Na, Ca, Mg, K, Cl and S) via ICP-OES. Quality control blanks were below detection limits. Standard reference material (NIST 2976 mussel tissue (freeze-dried)) was within 10% of expected concentrations.

To better understand performance differences of *N. triangulifer* in the different site waters, we used Visual MINTEQ, v. 3.0 (JP Gustafsson, Royal Institute of Technology, Department of Land and Water Resources Engineering, Stockholm, Sweden) to calculate the activity of all potential ionic species based on mean measured anion and cation concentrations. This is informative, because in more concentrated solutions, it may be necessary to account for the fact that ions behave as though their concentrations are lower than nominal or measured concentrations, i.e. an ion's 'activity' will probably be less than its concentration [32] (see [33]). For activity modelling, we input fixed pH values based on the average measured pH for each test.

(c) RNA isolation and quantitative polymerase chain reaction analysis for gene expression study

To compare the gene expression associated with sulfate stress, primers were designed based on a *de novo* assembly of compiled *N. triangulifer* cDNA sequence data (both 454 and Illumina platforms) resulting in approximately 23 000 contigs with associated bioinformatics. Based on these annotated contigs, we selected two genes of interest, a sulfate transporter and a Na-independent sulfate transporter, and developed probes for gene expression studies (table 3). *Tubulin* is served as a reference gene. Each gene of interest was inserted into a pCR2.1®-TOPO® TA vector (Life Technologies) expression vector and produced sequences that were independently confirmed. All primers were designed with IDTSciTools (http://www.idtdna.com/SciTools/SciTools.aspx) and were synthesized by Life Technologies, (Carlsbad, CA, USA).

For gene expression studies, *N. triangulifer* mature larvae were exposed to ASW amended with Mg/CaSO$_4$ and Na$_2$SO$_4$ (both containing 600 mg l^{-1} SO$_4$) for 24 h. Total RNA was isolated (two larvae pooled for one biological replicate) following the SV Total RNA Isolation System protocol (Promega, Madison, WI). First-strand cDNA was synthesized from the same amount of each total RNA by MultiScribe™ MuLV reverse transcriptase using random primers (Applied Biosystems, Carlsbad, CA, USA) and all thermocycling was done using a Bio-Rad iCycler (Bio-Rad, Hercules, CA). RT-qPCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) using default parameters: 2 min at 94°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Relative expression of each amplicon was calculated by the corrected delta–delta Ct method [34], with all expression normalized to *Tubulin* levels in initial control samples. Relative levels of *Tubulin* were confirmed to be approximately equal across all treatments. Results were plotted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). All treatments were compared to ASW (control group) and a Student's *t*-test was performed to analyse the differences of gene expression between treatments and control. All biological replicates for treatment groups and control group had $n = 6$.

3. Results

Measured sulfate concentrations were in good agreement with targeted values (mean and s.d. of $101 \pm 3\%$ of target values, after taking into consideration the sulfate in the site waters) (table 2). Control survival (% of larvae to successfully emerge to the subimago stage) in ASW was $82 \pm 2\%$, while survival in unmodified site water (site controls) was $76 \pm 2\%$ and $76 \pm 6\%$ in site A and B water controls, respectively. In site waters amended with SO$_4$ between 296 and 1000 mg l^{-1} SO$_4$, survival varied between 58 ± 12 and $80 \pm 12\%$. This variation was idiosyncratic and not systematically associated with changes in exposure concentration. However, at 1500 mg l^{-1} SO$_4$, survival decreased to $33 \pm 4\%$ and $29 \pm 6\%$ in site A and B waters, respectively (figure 1).

Mean subimago body masses were remarkably consistent across controls and SO$_4$ treatments with the exception of the 1500 mg l^{-1} SO$_4$ treatment group (figure 2). Relative to their respective site controls, the 1500 mg l^{-1} treated subimago body weights were reduced 16% and 22% in site A and B waters, respectively. In general, larvae seemed to grow better in the site A (control and amended) water than in site B counterpart treatments, but these differences were only significantly different ($p < 0.05$) for site controls, 296 mg l^{-1} and 1000 mg l^{-1} SO$_4$ treatments (Tukey's multiple comparison test).

Development time was significantly affected by sulfate exposures to 667 mg l^{-1} (site A), 1000 mg l^{-1} (both waters) and 1500 mg l^{-1} (both waters) (figure 3). Larvae that survived exposure to 1500 mg l^{-1} SO$_4$ took 33% (site A water) and 40% (site B water) longer to reach the subimago stage
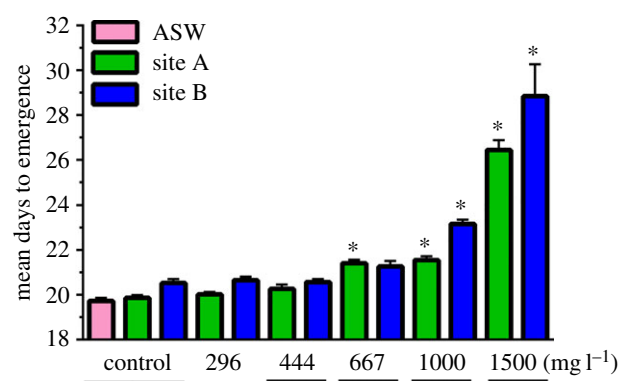
Table 3. List of primer sequences for qPCR gene expression studies.

gene name	accession number	amplicon	primer sequences
tubulin	MF463012	219	forward: 5′-ATGCCCTCTGACAAGACTGTTGGA-3′
			reverse: 5′-ATAGTGACCGCGAGCGTAGTTGTT-3′
sulfate transporter	MH549240	204	forward: 5′-TGATCATTACCGGCATCATCGGCT-3′
			reverse: 5′-TTTCCAGAATGGCGAGGATAGGCA-3′
Na-independent sulfate transporter	MH549239	181	forward: 5′-CTTCATTCCACGCACTACC-3′
			reverse: 5′-CACCACCATGCCGATTAG-3′
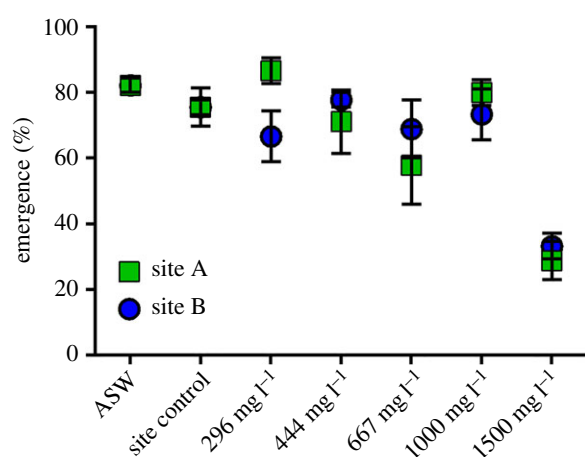
Figure 1. Emergence percentage of *N. triangulifer* subimagos reared in two site waters amended with SO_4 as a blend of $CaSO_4$ and $MgSO_4$. Each point represents the mean of three replicate rearing chambers each seeded with 15 larvae 1 day post egg hatch. (Online version in colour.)
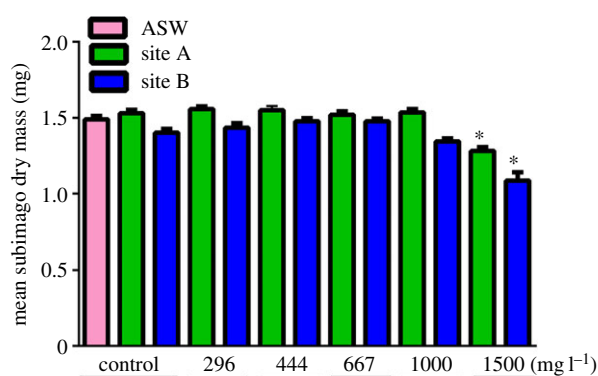
Figure 2. Mean of individual subimago weights (dry mass) emerging from different sulfate exposures.

Figure 3. Average time (days) required for completion of larval development in natural waters amended with SO_4 as a blend of $CaSO_4$ and $MgSO_4$.

than did their respective controls. Exposure to $1000 \, \mathrm{mg \, l^{-1}}$ prolonged development by 9% and 13% in site A and B waters, respectively. Exposure to $667 \, \mathrm{mg \, l^{-1}}$ (site A) prolonged development by 8%, whereas in site B water, this exposure did not significantly delay development.

Previous work examining the kinetics of $^{35}SO_4$ uptake as a function of concentration [30] showed expected Michaelis–Menten-type kinetics, but surprisingly did not fully saturate even at the highest SO_4 concentrations tested (figure 4a). However, this rapid SO_4 uptake in larvae did not result in appreciable changes in whole body S content in subimagos (figure 4b). Though statistically significant ($R^2 = 0.28$, $p = 0.0003$), there was little appreciable increase in whole body S

across four orders of magnitude of SO_4 exposure concentrations. Across all treatments, S content varied between 6.6 and $7.8 \, \mathrm{mg \, g^{-1}}$ on a dry weight basis, (mean $= 7.2 \pm 0.05$), suggesting that *N. triangulifer* strongly regulates S content.

Modelled sulfate activities in test treatments ranged from approximately 0.2 (controls) to approximately 5 mM in the highest test treatments in both waters (see the electronic supplementary material, table S1). In both waters, activities decreased as a per cent of concentration with increasing Ca and Mg in dilution water. For example, in the controls for both waters, free sulfate activity was approximately 80% of the measured concentration, whereas in the $1500 \, \mathrm{mg \, l^{-1}}$ treatments, free sulfate activity decreased to approximately 48% of the measured concentration. The slightly better performance of *N. triangulifer* in site A waters relative to site B was not directly attributable to differences in SO_4 activity, but both differences in activities and performance were relatively subtle.

To further our understanding of the osmoregulatory physiology of *N. triangulifer*, we examined the expression patterns of two genes that encode a sulfate transporter protein. Gene expression results showed that the expression level of sulfate transporter only reduced slightly under $MgSO_4$ and $CaSO_4$ but had a significant 74% reduction under Na_2SO_4 treatment ($p = 0.002$) (figure 5a). The expression level of the sodium-independent sulfate transporter gene, on the other hand, had a significant 55% reduction ($p = 0.0001$) under $MgSO_4$ and $CaSO_4$ treatment but no statistically significant differences under Na_2SO_4 treatment (figure 5b).

4. Discussion

The emergence of freshwater salinization as an ecological problem [1–3,5,11] requires that we rapidly fill gaps in our
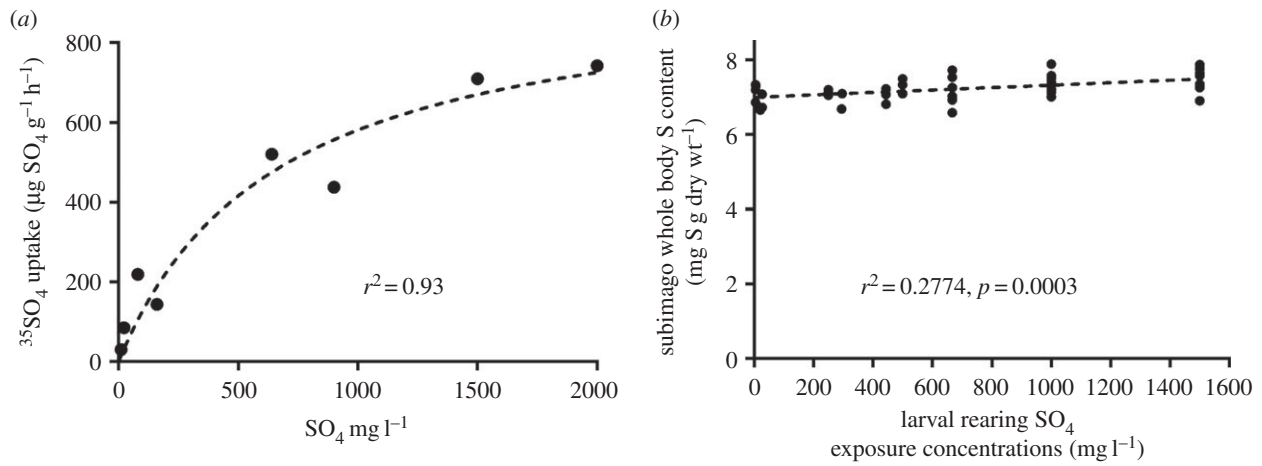
Figure 4. (a) $^{35}SO_4$ uptake rates in larvae of the mayfly *N. triangulifer* as a function of SO_4 concentrations (re-drawn from Scheibener *et al.* [30]). (b) Total body S concentrations in subimagos reared for their entire larval stage at different SO_4 concentrations.
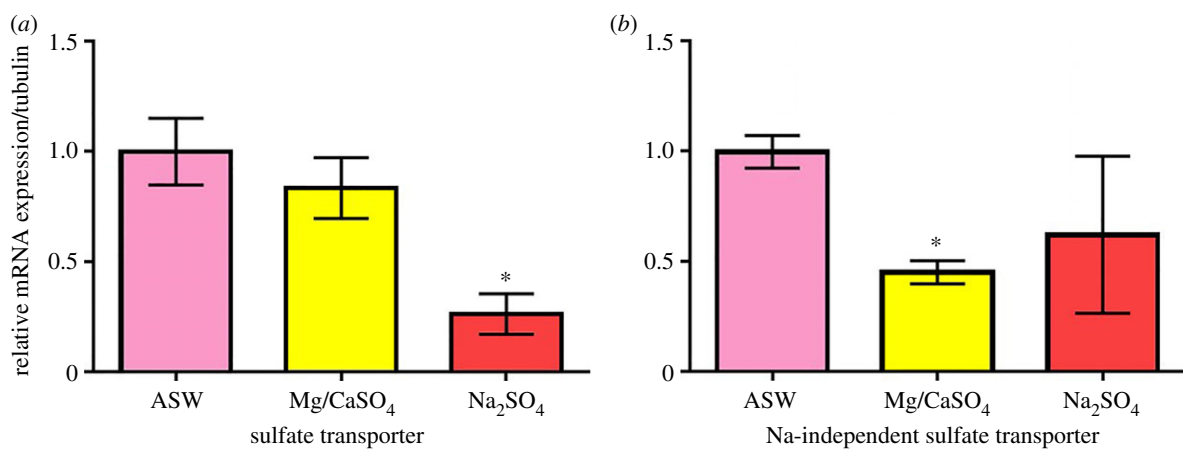

Figure 5. Relative mRNA expression of *N. triangulifer* larvae subjected to 24 h sulfate stress treatment. All data are normalized to control (ASW). Error bars represent standard deviations from the mean ($n = 6$). A Student's *t*-test was performed and an asterisk indicates significant difference compared to ASW control. (Online version in colour.)

understanding of how major ions affect aquatic life. It is important to acknowledge that mechanisms of major ion toxicity may differ among faunal groups, particularly in relation to evolutionary history. Insects dominate the ecology of running waters, and this alone should compel us to better understand how major ions shape their distributions and responses to changes in salinity regimes being reported around the globe. Moreover, the physiology of aquatic insects may be unique resulting from their history as secondarily aquatic species [35,36], and this should compel us to better understand fundamental physiological processes in relation to salinity.

Previous work [37] showed that major ion toxicity in the mayfly *N. triangulifer* could not be attributed to conductivity alone. Somehow, the ionic composition of the water mattered as waters high in sulfate, but low in sodium, were toxic to the mayfly, but when the ratios of SO_4 to Na were considerably lower, toxicity was not observed despite the high conductivity. Subsequent work identified Na as a modifier of acute SO_4 toxicity, with increasing Na associated with decreased SO_4 uptake and toxicity [30]. This finding is important, because in streams affected by surface coal mining, it is common to find elevated SO_4 against a low Na background.

In this paper, we evaluated the toxicity of SO_4 in two natural waters with relatively low Na. Sulfate exposures were created by amending these waters with a blend of Ca and Mg sulfate to maintain this low Na background. We anticipated that low Na waters might produce toxicity at lower SO_4 concentrations than those reported by Soucek & Dickinson [27] who used Na_2SO_4 and reported an EC20 for per cent emergence at 145 mg l^{-1} (95% CI 69–305). In our study, per cent emergence was not affected at 1000 mg l^{-1} SO_4, but was markedly reduced at 1500 mg l^{-1} SO_4.

There are a few possible explanations for the discrepancy between these results and those of the Soucek & Dickinson study [27]. First, our experimental temperatures varied between 23.3 and 24.8°C, while the Soucek & Dickinson study was conducted at 25°C, which is quite close to the edge of this species' thermal acclimation zone (see [38] and [39] for a discussion of the importance of temperature to mayfly growth and development). The Soucek & Dickinson study reports pre-egg laying fresh body weights of 2.5–2.6 mg per individual (wet weight), where our dry weights were typically 1.5 mg (approx. 6.8 mg wet weight based on a typical body water content of *N. triangulifer* of approx. 78%). Thus, there were substantial differences in larval growth in these studies that can be partially attributable to rearing temperature. Another strong possibility is that there are nutritional differences between the natural periphyton plate diet provided in the present study and the cultured

diatom diet provided in the Soucek & Dickinson study. A third and potentially important factor is that the use of concentration unit ($mg\,l^{-1}$) rather than ionic activities as a descriptor of toxicity may confound comparisons among studies. The $1500\,mg\,l^{-1}$ treatments in both waters of the present study had sulfate activities (5.06 and 4.86 mM for sites A and B, respectively) that approached the average 96 h median lethal sulfate activity (average of approx. 5.5 mM) in experiments with Na_2SO_4, $MgSO_4$ and K_2SO_4 in a study by Soucek et al. [40]. Additionally, the $1000\,mg\,l^{-1}$ treatment consistently caused delayed development in both waters in the present study had SO_4 activities that were only slightly higher than activity determined by Soucek & Dickinson [27] to cause developmental delay in their study. This difference in performance might be attributable to nutritional and thermal differences between the studies as described above. Nonetheless, it is apparent that using ionic activity as a descriptor of toxicity makes the results of the present study and the Soucek & Dickinson study more comparable than if concentration is used as a descriptor of toxicity.

Body weights were unaffected by SO_4 concentrations up to $1000\,mg\,l^{-1}$, but the development time required to achieve those weights was increased at $667\,mg\,l^{-1}$ SO_4. This observation is consistent with previous studies showing significant developmental delays associated with exposure to ion-rich waters [27,41]. These observations are suggestive of increased energetic demand associated with development in ion-rich waters in mayflies.

Scheibener et al. [30] previously characterized Michaelis–Menten-type uptake kinetics for SO_4 in N. triangulifer and showed that even at a very high SO_4 concentration, uptake of the ion was not saturated. To determine whether this situation could lead to 'self-poisoning' through excessive SO_4 accumulation in tissues, we analysed the total body S content of the subimagos that emerged during the study. We found that total body S was relatively stable across treatments, which suggests that N. triangulifer must be strongly regulating its SO_4 content by increasing SO_4 excretion rates commensurate with uptake rates. This finding is similar to previous observations of Na regulation in Hydropsyche sparna and Maccaffertium sp. [42], and related observation of the maintenance of haemolymph osmolality in the mayfly Austrophlebioides pusillus even at lethal salinities [43].

Together, these observations suggest that the energetic cost of regulation, rather than the loss of regulation, might drive the effects of elevated major ions on aquatic insects. What is intriguing about this proposition is that sensitivity to elevated major ions might be predictable based on how species regulate the uptake of a given ion when it is present at high concentrations. Species that maintain relatively low uptake rates might be expected to be less responsive to salinity increases. However, our knowledge of the specific ion transporters and how the expression and activities of these transporters are regulated remains virtually unknown.

Our identification of two distinct genes that respond differently to SO_4 exposures is a small first step towards understanding these processes. Both our sulfate transporter and Na-independent sulfate transporter genes are annotated using bioinformatics gene prediction methods. Both genes are suggested to encode proteins that belong to the sulfate permease (SulP) family, which mostly consists of inorganic anion uptake transporters or anion:anion exchange transporters [44]. While the sulfate transporter gene was also suggested as a Na-independent sulfate transporter gene in orthologues, experimental results in this study suggest the sulfate transporter gene expression is different than the Na-dependent sulfate transporter in N. triangulifer. Therefore, it is likely that the two genes encode different functional proteins. However, we posit that expression levels of the two genes may also vary in different tissues, and thus it is inconclusive for our data whether the two gene-encoded proteins have different functions or not. Na dependent or not, our data support the likely existence of sulfate transporters in N. triangulifer, and high sulfate treatments affect its gene expression levels. It remains unknown which tissues have more sulfate transporters and the differences between expression levels.

Finally, if salinity differentially challenges aquatic species via the energetic costs of regulation, then modifying factors such as temperature and nutritional availability may play huge roles in ultimately determining how communities and biodiversity change in the face of increasing salinization. If, for example, increasing temperature increases the rates of ion uptake commensurate with basic metabolic rates, the potential for salinity–temperature interactions are great. We might hypothesize that sensitivity to major ions might increase with increasing temperature. Similarly, individuals that are developing with access to sub-optimal nutrition (more oligotrophic conditions) might be considerably more sensitive to changes in salinity regimes than individuals developing with closer-to-optimal dietary resources.

References

1. Canedo-Arguelles M, Kefford BJ, Piscart C, Prat N, Schafer RB, Schulz CJ. 2013 Salinisation of rivers: an urgent ecological issue. Environ. Pollut. **173**, 157–167. (doi:10.1016/j.envpol.2012.10.011)

2. Canedo-Arguelles M et al. 2016 Saving freshwater from salts. Science **351**, 914–916. (doi:10.1126/science.aad3488)

3. Kaushal SS, Likens GE, Pace ML, Utz RM, Haq S, Gorman J, Grese M. 2018 Freshwater salinization syndrome on a continental scale. Proc. Natl Acad. Sci. USA **115**, E574–E583.

4. Kefford BJ, Hickey GL, Gasith A, Ben-David E, Dunlop JE, Palmer CG, Allan K, Choy SC, Piscart C. 2012 Global scale variation in the salinity sensitivity of riverine macroinvertebrates: eastern Australia, France, Israel and South Africa. PLoS ONE **7**, e35224. (doi:10.1371/journal.pone.0035224)

5. Kefford BJ, Buchwalter D, Canedo-Arguelles M, Davis J, Duncan RP, Hoffmann A, Thompson R. 2016 Salinized rivers: degraded systems or new habitats

for salt-tolerant faunas? *Biol. Lett.* **12**, 20151072. (doi:10.1098/rsbl.2015.1072)

6. Pond GJ, Passmore ME, Borsuk FA, Reynolds L, Rose CA. 2008 Downstream effects of mountaintop coal mining: comparing biological conditions using family and genus-level macroinvertebrate bioasessment tools. *J. North Am. Benthol. Soc.* **127**, 717–737. (doi:10.1899/08-015.1)

7. Karraker NE, Gibbs JP, Vonesh JR. 2008 Impacts of road deicing salt on the demography of vernal pool-breeding amphibians. *Ecol. Appl.* **18**, 724–734. (doi:10.1890/07-1644.1)

8. Clements WH, Kotalik C. 2016 Effects of major ions on natural benthic communities: an experimental assessment of the US Environmental Protection Agency aquatic life benchmark for conductivity. *Freshw. Sci.* **35**, 126–138. (doi:10.1086/685085)

9. Williams WD. 2001 Salinization: unplumbed salt in a parched landscape. *Water Sci. Technol.* **43**, 85–91. (doi:10.2166/wst.2001.0186)

10. Williams WD. 2001 Anthropogenic salinization of inland waters. *Hydrobiologia* **466**, 329–337. (doi:10.1023/A:1014598509028)

11. Kaushal SS, Groffman PM, Likens GE, Belt KT, Stack WP, Kelly VR, Band LE, Fisher GT. 2005 Increased salinization of fresh water in the northeastern United States. *Proc. Natl Acad. Sci. USA* **102**, 13 517–13 520. (doi:10.1073/pnas.0506414102)

12. Kaushall SS et al. 2017 Human-accelerated weathering increases salinization, major ions, and alkalinization in fresh water across land use. *Appl. Geochem.* **83**, 121–135. (doi:10.1016/j.apgeochem.2017.02.006)

13. Entrekin S, Evans-White M, Hagenbuch E. 2011 Rapid expansion of natural gas development poses a threat to surface waters. *Front. Ecol. Environ.* **9**, 503–511. (doi:10.1890/110053)

14. Lauer NE, Harkness JS, Vengosh A. 2016 Brine spills associated with unconventional oil development in North Dakota. *Environ. Sci. Technol.* **50**, 5389–5397. (doi:10.1021/acs.est.5b06349)

15. Mosley LM. 2017 Drought impacts on the water quality of freshwater systems; review and integration. *Earth Sci. Rev.* **140**, 203–214. (doi:10.1016/j.earscirev.2014.11.010)

16. Sweeney BW, Vannote RL. 1984 Influence of food quality and temperature on life-history characteristics of the parthenogenetic mayfly, *Cloeon triangulifer. Freshw. Biol.* **14**, 621–630. (doi:10.1111/j.1365-2427.1984.tb00181.x)

17. Sweeney BW, Funk DH, Standley LJ. 1993 Use of the stream mayfly *Cloeon triangulifer* as a bioassay organism—life-history response and body burden following exposure to technical Chlordane. *Environ. Toxicol. Chem.* **12**, 115–125. (doi:10.1002/etc.5620120113)

18. Xie L, Funk DH, Buchwalter DB. 2010 Trophic transfer of Cd from natural periphyton biofilms to the grazing mayfly *Centroptilum triangulifer* in a life cycle test. *Environ. Pollut* **158**, 272–277. (doi:10.1016/j.envpol.2009.07.010)

19. Xie L, Buchwalter DB. 2011 Cadmium exposure route affects antioxidant responses in the mayfly

Centroptilum triangulifer. Aquat. Toxicol. **105**, 199–205. (doi:10.1016/j.aquatox.2011.06.009)

20. Conley JM, Funk DH, Buchwalter DB. 2009 Selenium bioaccumulation and maternal transfer in the mayfly *Centroptilum triangulifer* in a life-cycle, periphyton-biofilm trophic assay. *Environ. Sci. Technol.* **43**, 7952–7957. (doi:10.1021/es9016377)

21. Conley JM, Funk DH, Cariello NJ, Buchwalter DB. 2011 Food rationing affects dietary selenium bioaccumulation and life cycle performance in the mayfly *Centroptilum triangulifer. Ecotoxicology* **20**, 1840–1851. (doi:10.1007/s10646-011-0722-1)

22. Conley JM, Funk DH, Hesterberg DH, Hsu LC, Kan J, Liu YT, Buchwalter DB. 2013 Bioconcentration and biotransformation of selenite versus selenate exposed periphyton and subsequent toxicity to the mayfly *Centroptilum triangulifer. Environ. Sci. Technol.* **47**, 7965–7973. (doi:10.1021/es400643x)

23. Conley JM, Watson AT, Xie L, Buchwalter DB. 2014 Dynamic selenium assimilation, distribution, efflux, and maternal transfer in *Japanese medaka* fed a diet of se-enriched mayflies. *Environ. Sci. Technol.* **48**, 2971–2978. (doi:10.1021/es404933t)

24. Kim KS, Funk DH, Buchwalter DB. 2012 Dietary (periphyton) and aqueous Zn bioaccumulation dynamics in the mayfly *Centroptilum triangulifer. Ecotoxicology* **21**, 2288–2296. (doi:10.1007/s10646-012-0985-1)

25. Wesner JS, Walters DM, Schmidt TS, Kraus JM, Stricker CA, Clements WH, Wolf RE. 2017 Metamorphosis affects metal concentrations and isotopic signatures in a mayfly (*Baetis tricaudatus*): implications for the aquatic-terrestrial transfer of metals. *Environ. Sci. Technol.* **51**, 2438–2446. (doi:10.1021/acs.est.6b05471)

26. Wesner JS, Kraus JM, Schmidt TS, Walters DM, Clements WH. 2014 Metamorphosis enhances the effects of metal exposure on the mayfly, *Centroptilum triangulifer. Environ. Sci. Technol.* **48**, 10 415–10 422. (doi:10.1021/es501914y)

27. Soucek DJ, Dickinson A. 2015 Full-life chronic toxicity of sodium salts to the mayfly *Neocloeon triangulifer* in tests with laboratory cultured food. *Environ. Toxicol. Chem.* **34**, 2126–2137. (doi:10.1002/etc.3038)

28. Struewing KA, Lazorchak JM, Weaver PC, Johnson BR, Funk DH, Buchwalter DB. 2015 Part 2: sensitivity comparisons of the mayfly *Centroptilum triangulifer* to *Ceriodaphnia dubia* and *Daphnia magna* using standard reference toxicants; NaCl, KCl and CuSO4. *Chemosphere* **139**, 597–603. (doi:10.1016/j.chemosphere.2014.04.096)

29. Weaver PC, Lazorchak JM, Struewing KA, DeCelles SJ, Funk DH, Buchwalter DB, Johnson BR. 2015 Part 1: laboratory culture of *Centroptilum triangulifer* (Ephemeroptera: Baetidae) using a defined diet of three diatoms. *Chemosphere* **139**, 589–596. (doi:10.1016/j.chemosphere.2014.04.092)

30. Scheibener S, Conley JM, Buchwalter D. 2017 Sulfate transport kinetics and toxicity are modulated by sodium in aquatic insects. *Aquat. Toxicol.* **190**, 62–69. (doi:10.1016/j.aquatox.2017.06.027)

31. Mount DR, Gulley DD, Hockett JR, Garrison TD, Evans JM. 1997 Statistical models to predict the toxicity of

major ions to *Ceriodaphnia dubia, Daphnia magna* and *Pimephales promelas* (fathead minnows). *Environ. Toxicol. Chem.* **16**, 2009–2019. (doi:10.1897/1551-5028(1997)016<2009:SMTPTT>2.3.CO;2)

32. Snoeyink VL, Jenkins D. 1980 *Water chemistry*. New York, NY: John Wiley and Sons.

33. Erickson RJ, Mount DR, Highland TL, Hockett JR, Hoff DJ, Jenson CT, Norberg-King TJ, Peterson KN. 2018 The acute toxicity of major ion salts to *Ceriodaphnia dubia*. III. Modeling the toxicity of major ion mixtures. *Environ. Toxicol. Chem.* **37**, 247–259.

34. Pfaffl MW. 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acid. Res.* **29**, e45.

35. Misof B et al. 2014 Phylogenomics resolves the timing and pattern of insect evolution. *Science* **346**, 763–767. (doi:10.1126/science.1257570)

36. Kristensen NP. 1981 Phylogeny of insect orders. *Annu. Rev. Entomol.* **26**, 135–157. (doi:10.1146/annurev.en.26.010181.001031)

37. Kunz JL, Conley JM, Buchwalter DB, Norberg-King TJ, Kemble NE, Wang N, Ingersoll CG. 2013 Use of reconstituted waters to evaluate effects of elevated major ions associated with mountaintop coal mining on freshwater invertebrates. *Environ. Toxicol. Chem.* **32**, 2826–2835. (doi:10.1002/etc.2391)

38. Kim KS, Chou H, Funk DH, Jackson JK, Sweeney BW, Buchwalter DB. 2017 Physiological responses to short-term thermal stress in mayfly (*Neocloeon triangulifer*) larvae in relation to upper thermal limits. *J. Exp. Biol.* **220**, 2598–2605. (doi:10.1242/jeb.156919)

39. Sweeney BW, Funk DH, Jackson JK, Camp AA, Buchwalter D. 2018 Why a mayfly *Cloeon dipterum* (Ephemeroptera: Baetidae) gets smaller as temperatures warm. *Freshw. Sci.* **37**, 64–81. (doi:10.1086/696611)

40. Soucek DJ, Mount DR, Dickinson A, Hockett JR. 2018 Influence of dilution water ionic composition on acute major ion toxicity to the mayfly *Neocloeon triangulifer. Environ. Toxicol. Chem.* **37**, 1330–1339. (doi:10.1002/etc.4072)

41. Johnson BR, Weaver PC, Nietch CT, Lazorchak JM, Struewing KA, Funk DH. 2015 Elevated major ion concentrations inhibit larval mayfly growth and development. *Environ. Toxicol. Chem.* **34**, 167–172. (doi:10.1002/etc.2777)

42. Scheibener SA, Richardi VS, Buchwalter DB. 2016 Comparative sodium transport patterns provide clues for understanding salinity and metal responses in aquatic insects. *Aquat. Toxicol.* **171**, 20–29. (doi:10.1016/j.aquatox.2015.12.006)

43. Dowse R, Palmer CG, Hills K, Torpy F, Kefford BJ. 2017 The mayfly nymph *Austrophlebioides pusillus* Harker defies common osmoregulatory assumptions. *R. Soc. open sci.* **4**, 160520. (doi:10.1098/rsos.160520)

44. Piłsyk S, Paszewski A. 2009 Sulfate permeasesphylogenetic diversity of sulfate transport. *Acta Biochim. Pol.* **56**, 375–384.