Chemosphere 139 (2015) 597–603

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Part 2: Sensitivity comparisons of the mayfly *Centroptilum triangulifer* to *Ceriodaphnia dubia* and *Daphnia magna* using standard reference toxicants; NaCl, KCl and $CuSO_4$

Katherine A. Struewing [a,1], James M. Lazorchak [b,*], Paul C. Weaver [a], Brent R. Johnson [b], David H. Funk [c], David B. Buchwalter [d]

[a] The McConnell Group, 26 W. MLK Blvd., Cincinnati, OH 45268, United States
[b] U.S. Environmental Protection Agency, 26 W. MLK Blvd., Cincinnati, OH 45268, United States
[c] Stroud Water Research Center, Avondale, PA, United States
[d] North Carolina State University, Raleigh, NC, United States

HIGHLIGHTS

- New mayfly test species for use in toxicity tests more sensitive than standard test species.
- Procedures for conducting acute and chronic tests with a sensitive mayfly are presented.
- Testing with *Centriptilum triangulifer* may afford more protection to EPT species.

ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 14 April 2014
Accepted 24 April 2014
Available online 2 June 2014

Handling Editor: A. Gies

Keywords:
Mayfly
Comparative toxicity
NaCl
KCl
$CuSO_4$

ABSTRACT

Criteria for establishing water quality standards that are protective for 95% of the native species are generally based upon laboratory toxicity tests. These tests utilize common model organisms that have established test methods. However, for invertebrates these species represent mostly the zooplankton community and are not inclusive of all taxa. In order to examine a potential under-representation in emerging aquatic invertebrates the US Environmental Protection Agency has cultured a parthenogenetic mayfly, *Centroptilum triangulifer* (Ephemeroptera: Baetidae). This study established a 48 h acute and a 14-day short-term chronic testing procedure for *C. triangulifer* and compared its sensitivity to two model invertebrates, *Ceriodaphnia dubia* and *Daphnia magna*. Toxicity tests were conducted to determine mortality and growth effects using standard reference toxicants: NaCl, KCl and $CuSO_4$. In 48-h acute tests, the average LC50 for the mayfly was 659 mg L^{-1} NaCl, 1957 mg L^{-1} KCl, and 11 μg L^{-1} $CuSO_4$. IC25 values, using dry weight as the endpoint, were 228 mg L^{-1} NaCl, 356 mg L^{-1} KCl and 5 μg L^{-1} $CuSO_4$. *C. triangulifer* was the most sensitive species in NaCl acute and chronic growth tests. At KCl concentrations tested, *C. triangulifer* was less sensitive for acute tests but was equally or more sensitive than *C. dubia* and *D. magna* for growth measurements. This study determined *C. triangulifer* has great potential and benefits for use in ecotoxicological studies.

Published by Elsevier Ltd.

1. Introduction

Laboratory toxicity tests with aquatic organisms have been used for decades to assess the hazard of chemicals in freshwater environments and as a basis for developing water quality criteria. All most all laboratory toxicity tests are conducted using a relatively small number of model organisms, which have been preferred for reasons such as their ease of culturing in a laboratory, life history traits (e.g., rapid life cycle), or limited variability between individuals. Common model organisms include the fishes *Pimephales promelas* and *Oncorhynchus mykiss*, the cladocerans *Ceriodaphnia dubia* and *Daphnia magna*, the amphipod *Hyalella azteca*, and the midge *Chironomus dilutus*, among others. These aquatic species have been used extensively for acute and chronic toxicity testing and have standardized methods published by the US Environmental Protection Agency (EPA) (United States

* Corresponding author. Address: U.S. EPA ORD, 26 W Martin Luther King Drive, Cincinnati, Ohio 45268, United States. Tel.: +1 513 569 7076.
E-mail address: Lazorchak.jim@epa.gov (J.M. Lazorchak).
[1] Current address: Arcadis, 4665 Cornnell Rd, Cincinnati, OH 45241, United States.

http://dx.doi.org/10.1016/j.chemosphere.2014.04.096
0045-6535/Published by Elsevier Ltd.

Environmental Protection Agency, 2002a,b), American Society for Testing and Materials (ASTM), and others.

There are many benefits to using a model organism, including the ability to compare results from the literature, but there may also be disadvantages. Developing water quality criteria that are protective of all native fauna is an important goal of aquatic toxicity studies, but the use of model organisms that are restricted to a few taxa may miss some of the more sensitive species. For example, Brix et al. (2005) argue that toxicity data for metals under-represent aquatic insects and over-represent cladocerans.

The number of toxicity tests using aquatic insects has begun to increase, especially within the order Ephemeroptera (mayflies) (Diamond et al., 1992; Lowell et al., 1995; Irving et al., 2003; Mebane et al., 2008; Echols et al., 2010). *Centroptilum triangulifer* (Ephemeroptera: Baetidae) was previously identified as *Cloeon triangulifer* by McDunnough in 1931 (McDunnough, 1931). The species is found in marginal streams and slow flowing aquatic systems throughout eastern North America (Gibbs et al., 1973). Funk et al. (2006) used genetic and morphological examinations to describe differences between *C. triangulifer*, which is obligatory parthenogenetic, and *Centroptilum alamance*, which is a similar species that reproduces sexually. *C. triangulifer* females produce clonal eggs and embryonic development is temperature dependent, averaging 6 d until first hatch at 25 °C (Sweeney and Vannote, 1984). The relative short life cycle (~30 d) and clonal reproduction makes *C. triangulifer* a good candidate for toxicology studies.

Studies using *C. triangulifer*, generally indicate this species is sensitive to certain pesticides, metals, and non-metals (Sweeney et al., 1993; Standley et al., 1994; Conley et al., 2009; Xie et al., 2010; Xie and Buchwalter, 2011). Hassell et al. (2006) determined that survival of *Centroptilum* sp. was decreased when salinity (as measured by conductivity) was equal or greater than 5.0 μS cm^{-1} which was lower than the *Cloeon* sp. tested in the same study. In these studies, authors have used field collected or laboratory cultured organisms. The food used for laboratory culture was provided in the form of acrylic plates that had been allowed to colonize with biofilm while being held under flowing natural stream water or soften condition leaves (Sweeney et al., 1993; Standley et al., 1994; Funk et al., 2006; Conley et al., 2009; Xie et al., 2010). The plates or leaves were then placed into culture or test containers. This approach may have shortcomings for standardizing test methods, including variability in the community or species composition and density of the biofilm, and potential introduction of toxicants or pathogens from ambient stream water. In addition, this method is not practical for those without access to a suitable stream.

An alternate means of maintaining mayfly cultures has been developed (Weaver et al., accepted for publication). This new culture method will help standardize culturing techniques, making *C. triangulifer* more easily cultured for use in testing. Now that culturing techniques have been standardized for this new organism, there is a need for standardized toxicity testing procedures. The establishment of a uniform testing procedure would increase the usefulness of *C. triangulifer* as a bioassay species. As studies utilizing mayflies have increased and a convenient culturing method has been documented, this study aims to establish standardized acute and chronic test methods for *C. triangulifer*.

This study compares results of *C. triangulifer* acute and chronic bioassays to parallel tests conducted with the common model organisms *C. dubia* and *D. magna*. The sensitivities of each of these three species are examined using standard reference toxicants. Comparing the sensitivities of the mayfly to the cladocerans will help to discern the usefulness of *C. triangulifer* in future toxicology studies.

2. Materials and methods

2.1. Test waters

Three waters were used, two for food preparation and culturing, and a third for toxicity testing. The first water, used only for food preparation, was Millipore Super-Q® system (SQ) water, produced from a system using 4 cartridges: an activated carbon filter, two ion exchange filters, and an Organex-Q filter. The second water was dechlorinated tap water (Lab-line); an in-house water which is a carbon filtered and dechlorinated tempered (24–26 °C) tap water. Lab-line water was supplemented with liquid calcium chloride to reach a hardness of 160–200 mg L^{-1} as measured by $CaCO_3$. Lab-line was used for culturing all three species of organisms. The third water, used for toxicity testing, was Moderately Hard Reconstituted Water (MHRW) (United States Environmental Protection Agency, 2002a) (hardness of 80–100 mg L^{-1} and alkalinity of 60–70 mg L^{-1} as $CaCO_{3)}$.

2.2. Test animals

2.2.1. C. triangulifer cultures

C. triangulifer cultures originated from eggs (SWRC clone WCC-2) obtained from the Stroud Water Research Center (Avondale, PA, http://stroudcenter.org/) in 2010 and cultured at AWBERC. Cultures were maintained at a constant temperature of 25 °C with a 16:8 H light to dark photoperiod (Weaver et al., accepted for publication).

After adults emerged (25–30 d at 25 °C), they were removed from the culturing container and placed into an empty jar. The adults were individually weighed on a Toledo Mettler® Scale to the nearest 0.00001 g in order to estimate egg production using a formula previously developed by Weaver et al. (accepted for publication). Each adult was held by the wings using forceps over a 25-mL vial of Lab-line and the abdomen lowered until it made contact with surface water. At that point, eggs were released rapidly. Separate vials were used to collect eggs from each adult. Depending on need, *C. triangulifer* eggs were either stored at 25 °C for use upon hatching (2–3 d) or stored at 4 °C for no more than 4 months. If stored at 4 °C, eggs were moved to a 25 °C incubator three to four days prior to test initiation. Methods for collection of adults and eggs were adapted from procedures from Stroud Water Research Center.

Food was added to each vial in the amount of 0.3 mL of the diatom mixture prior to hatching. The mixture had equal volumes of each species with a cumulative concentration of 74 mg dry weight (±5%) of diatoms/50 mL Lab-line water. Eggs were monitored for hatching daily and nymphs were discarded when necessary to ensure animals were <24 h old at time of test setup. Each test used a random pool of 2 or 3 vials of eggs (from 2 or 3 adults).

2.2.2. C. dubia cultures

C. dubia test organisms were cultured and maintained using standard techniques (United States Environmental Protection Agency, 2002a,b). Beakers of all-female cultures were restarted weekly, but staggered to ensure there were adults old enough to reproduce every day. Less than 24-h old *C. dubia* young were collected from the culture and held in 1 L MHRW with 2 mL *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), at a concentration of 100 million cells/mL added as a food source prior to test initiation.

2.2.3. D. magna cultures

D. magna were also cultured and maintained according to standard methods (United States Environmental Protection Agency,

2002a, b). Cultures were fed 3 mL *P. subcapitata* and 3 mL blended alfalfa daily. Prior to acute or chronic test setup, *D. magna* young that were <24 h were collected from cultures and held in 1 L MHRW with 3 mL *P. subcapitata*.

2.3. Bioassay

Two salts and one metal were used as reference toxicants in this study. The two salts chosen were potassium chloride (KCl) (Fisher Scientific, laboratory grade, CAS 7447-40-7) and sodium chloride (NaCl) (Fisher Scientific, enzyme grade, CAS 7647-14-5). An extensive record of toxicity tests using KCl on *C. dubia* and *D. magna* has been maintained for years at AWBERC (United States Environmental Protection Agency, 2002a,b). The metal chosen for this study was copper (as $CuSO_4$) (Fluka, laboratory grade, CAS7758998), which is used as a standard reference toxicant by the U.S. EPA (United States Environmental Protection Agency, 2002a,b). Little data on copper toxicity currently exists for mayflies, but one study by Brinkman and Johnston (2008) examined acute toxicity testing of the Heptageniidae mayfly *Rhithrogena hageni* and reported a LC50 of 0.137 mg L^{-1} after 96 h at 12 °C.

The $CuSO_4$ stock solution was made by adding 0.982 g $CuSO_4$ into 250 mL SQ to create 1000 mg L^{-1} Cu++. When preparing $CuSO_4$ testing solutions, an aliquot of stock solution was added to 1 L MHRW to create the desired concentration level. Stock solutions were verified by ICP analyses and were within 80% of nominal. NaCl and KCl were added in salt form to MHRW when making testing concentrations. All test concentrations were prepared by serial dilutions of the highest concentrations. Concentrations were dispensed into 30 mL plastic cups using a sterile syringe and placed in an environmental incubator to reach the test temperature parameter of 25 °C.

The serial concentrations of NaCl used for acute testing ranged from 250.0 to 8000.0 mg L^{-1}. Concentrations for NaCl chronic testing were determined by a preliminary range finder and were from 78.1 to 1250.0 mg L^{-1} for *C. triangulifer*, 156.3–2500.0 mg L^{-1} for *C. dubia* and 312.5–5000.0 mg L^{-1} for *D. magna*. The treatment concentrations for acute KCl bioassays ranged from 62.5 to 2000.0 mg L^{-1}. During chronic KCl tests, the concentration range was 125.0–2000.0 mg L^{-1} for *C. triangulifer* and 62.5–1000.0 mg L^{-1} for both *C. dubia* and *D. magna*. Treatment concentrations for acute $CuSO_4$ bioassays ranged from 2.5 to 80.0 μg L^{-1}. Finally, in chronic $CuSO_4$ tests the concentrations ranged from 1.3 to 80.0 μg L^{-1} for *C. triangulifer*, 2.5–160.0 μg L^{-1} for *C. dubia* and 5.0–80.0 μg L^{-1} for *D. magna*.

Chemical analyses were not conducted due to past verification that nominal values were within 80% of measured values. Furthermore, measured chemistries were not performed since the purpose of the study was to compare relative sensitivity across the test species using the same solutions.

2.4. Acute bioassays

Acute bioassays were conducted for 48 h. The endpoint was mortality, expressed as an LC50 (lethal concentration for 50% of the population). When possible, tests comparing species were run simultaneously and with the same dilution water to limit variations in conditions. Procedures for acute testing were from U.S. EPA (United States Environmental Protection Agency, 2002a) acute toxicity test methods, except feeding which was modified as described below.

Preliminary data indicated *C. triangulifer* did not meet acceptable survival criterion without feeding even in the acute tests. Non-fed <24-h mayflies had 11% survival after 48 h in MHRW, while <24-h mayflies that were feed 0.2 mL diatom mixture [74(±5%)mg/50 mL] prior to the test, but not during, had 56% survival. To further ascertain the minimal required feeding, another 48 h test was conducted feeding <24-h mayflies 0.1 mL, 0.05 mL or 0.03 mL of diatom mixture daily. Survival results were 100%, 89%, and 83% respectively. Since 0.1 mL of diatom mix daily was the only feeding regimen to pass survival criteria it was used in all acute testing.

C. dubia and *D. magna* acute bioassays do not require feeding (United States Environmental Protection Agency, 2002a), but to eliminate the possible interaction effects of food and toxicants all acute tests were fed 0.1 mL diatom mixture regardless of the test species. Organisms in the acute tests were fed twice during the test at initial setup (0-h) and after water renewal (24-h).

Test solutions were renewed and mortality was recorded after 24 h *C. dubia* and *D. magna* were counted and then carefully pipetted from the test board into cups of fresh solution on another board. Survival of *C. triangulifer* was determined using a dissecting stereomicroscope. An 80% water renewal was performed. Water renewal was performed by placing the tip of a 12.5 mL syringe below the surface of the water while observing under the stereomicroscope. Using a syringe, 12.5 mL of solution was slowly extracted without removing the animals. Then 12.5 mL of fresh testing water was added carefully to avoid injuring the organism.

2.5. Chronic bioassays

Due to variations in life cycles, the duration, conditions, and end point of chronic tests varied for each test species. A chronic method for *C. dubia* and sub-chronic *D. magna* method were already established from U.S. EPA Chronic Methods (United States Environmental Protection Agency, 2002b) and Lazorchak et al. (2009) respectively. As previous chronic methods for *C. triangulifer* were not established, test duration, feeding, and endpoints were established as described below. Initial and final measurements for temperature, DO, conductivity and pH were recorded daily for testing solution at each concentration.

2.5.1. C. triangulifer

Chronic studies for *C. triangulifer* were 14 d in duration. This test length was chosen because it was about 50% of their larval life cycle and provides adequate time for measurable growth to occur. Since *C. triangulifer* can be cannibalistic in limited space or nutrients (data not shown), only one animal was placed per 30-mL testing cup. Each cup contained 15 mL of solution with 10 replicates per concentration. Food quantity chosen was 0.2 mL daily, which was double the minimal food requirement used in acute testing. As the organisms grew in size, food quantity was increased from 0.2 mL to 0.4 mL at day 10. Excess diatoms were always visible in testing cups. Daily water changes were conducted using the same procedure described in acute testing.

The non-lethal endpoints for *C. dubia* (reproduction) and *D. magna* (growth) have already been established for chronic tests (United States Environmental Protection Agency, 2002b; Lazorchak et al., 2009). Growth of *C. triangulifer* was recorded using three separate criteria: head capsule width, body length, and weight. These three criteria were used in order to determine which one was the most sensitive indicator of growth. After 14 d mayflies were transferred individually using a pipette to an aluminum weigh pan containing enough water to keep the mayfly totally submerged and mobile. The weigh pans were placed under a 25× magnification stereomicroscope with attached digital camera. An image of each organism was taken using Image-Pro Plus® v.7.1 software. Software analysis was used to measure the head capsule (distance between outer edge of eyes) and body length (distance from tip of head to end of abdomen) to the nearest 0.001 mm. After images were taken, the water was removed using a fine tip pipette and weigh pans were transferred to a drying oven (60 °C). After

24 h, the pans were removed from the oven and organisms weighed on a Cahn® microbalance to the nearest 0.0001 mg.

2.5.2. C. dubia

C. dubia chronic bioassays lasted 7 d, as described in the U.S. EPA methods manual for chronic testing of fresh water invertebrates (United States Environmental Protection Agency, 2002b).

2.5.3. D. magna

Methods for the 4-d chronic *D. magna* test were taken from Lazorchak et al. (2009).

2.6. Statistical analysis

For all tests and species, the LC50 was established using the Trimmed Spearman–Karber (TSK) method (version 1.5), which adjusts for control mortality. Animals that were accidentally killed or lost (via spillage or cracked cup) were not included in TSK analysis. The IC25 or inhibitory concentration via a 25% reduction in a biological function was calculated for all chronic tests using the Linear Interpolation Method for Sublethal Toxicity (version 2.0). The IC25 was established using weight (*D. magna* and *C. triangulifer*), length and head capsule width (*C. triangulifer*) or offspring production (*C. dubia*).

For each test, survival, growth and/or reproductive endpoint differences within a species were analyzed using an ANOVA with post-Tukey comparisons ($p \leqslant 0.05$) in Systat 11 (Systat® Software, San Jose CA). Mean LC50s (for acute tests) and IC25s (for chronic tests) were compared between species for each toxicant by ANOVA with post-Tukey comparisons ($p \leqslant 0.05$) as well.

3. Results

Acute and chronic *C. triangulifer* bioassays produced over 90% survival in the controls for all tests, surpassing the test criteria. While the chronic test duration was not a full life study, 14 d was ample time for differential growth effects to occur when present. Test Conditions for DO, Temperature, pH, and Conductivity are provided in Supplemental Information.

3.1. NaCl

3.1.1. Acute tests

Three acute tests were conducted for each species using NaCl. Results from the NaCl acutes tests are displayed for each species as dose–response curves in supplement Fig. 1S. ANOVA indicated that the mean LC50 from each species was significantly different from the other (F{2} = 89.753, $p = 0.003$,) (Fig. 1). Post-Tukey comparisons indicate that *C. triangulifer*'s LC50 (658.7 mg L^{-1}) is significantly lower than *C. dubia*'s LC50 (2504.3 mg L^{-1}) ($p = 0.003$) and *D. magna*'s LC50 (4868.7 mg L^{-1}) is significantly higher than both *C. dubia* and *C. triangulifer* ($p = 0.001$) [NaCl acute mortality endpoint sensitivity: *C. triangulifer* ≫ *C. dubia* > *D. magna*].

3.1.2. Chronic tests

Chronic testing with NaCl was repeated 3 times for each species. ANOVA indicated there were significant differences between the mean LC50s (F{2} = 110.725, $p < 0.001$) (Fig. 1). Further post-Tukey comparisons show that mean LC50s for *C. triangulifer* (833.1 mg L^{-1}) and *C. dubia* (1571.3 mg L^{-1}) were not significantly different from each other ($p = 0.055$). Both were different from *D. magna* (4312.0 mg L^{-1}) ($p < 0.001$) [NaCl chronic mortality endpoint sensitivity: *C. triangulifer* = *C. dubia* > *D. magna*].

Growth data from the mayfly chronic tests indicated that weight was more sensitive than head capsule width or body length. The average IC25 for weight was 228.8 (±193.8) mg L^{-1}, head capsule width was 368.5 (±195.3) mg L^{-1} and body length was 360.0 (±192.8) mg L^{-1}. Although not statistically significant, weight was consistently the most sensitive growth parameter for *C. triangulifer*. The average IC25 for weight was at least 36% lower than the second lowest parameter in all toxicant tests. For the duration of the test, weight was used as the IC25 for analysis between species for all three toxicants.

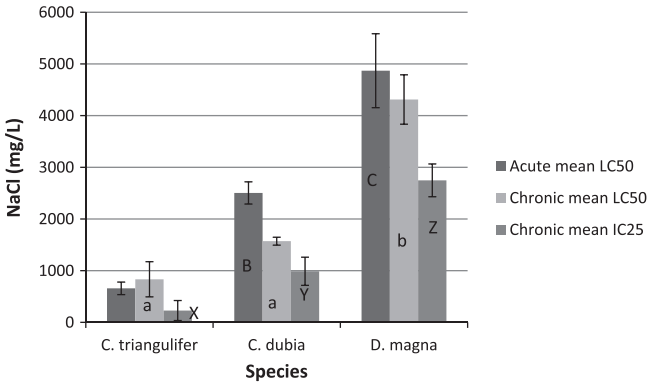

Fig. 1. Results from NaCl (mg L^{-1}) acute and chronic tests for all three species. Acute endpoint is depicted as mean LC50 while chronic endpoints include both the mean LC50 and mean IC25 results. ANOVA with post-Tukey results indicate differences between species ($p \leqslant 0.05$). Different letters within a test endpoint indicate a significant difference between mean values. Error bars represent 95% confidence intervals.

Mean IC25s for each species differed significantly from each other in an ANOVA (F{2} = 99.41, $p < 0.001$) (Fig. 2). *C. dubia* mean IC25 (988.9 mg L^{-1}) was significantly greater than *C. triangulifer* mean IC25 (228.8 mg L^{-1}) in post-Tukey comparisons ($p = 0.010$). *D. magna* mean IC25 (2747.2 mg L^{-1}) value was significantly greater than both *C. triangulifer* and *C. dubia* ($p < 0.001$) [NaCl growth/reproduction endpoint sensitivity: *C. triangulifer* (weight) > *C. dubia* (reproduction) > *D. magna* (weight)].

3.2. KCl

3.2.1. Acute tests

Dose–response curves from triplicate acute KCl tests are displayed in Fig. 2S. ANOVA of mean acute LC50s indicated significant differences between species (F{2} = 44.608, $p < 0.001$) (Fig. 2). The

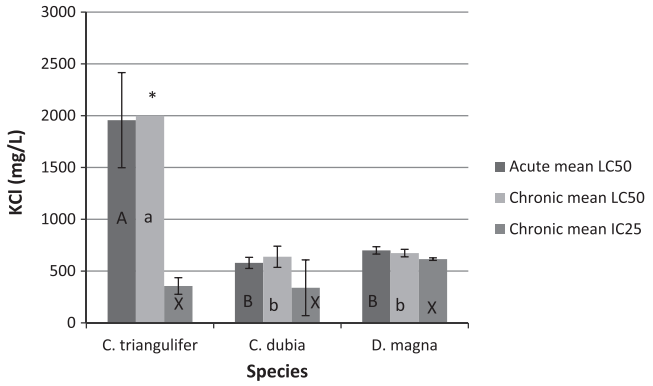

Fig. 2. Results from KCl (mg L^{-1}) acute and chronic tests for all three species. Acute endpoint is depicted as mean LC50 while chronic endpoints include both the mean LC50 and mean IC25 results. ANOVA with post-Tukey results indicate differences between species ($p \leqslant 0.05$). Different letters within a test endpoint indicate a significant difference between mean values. Error bars represent 95% confidence intervals. *LC50 above highest concentration tested (2000 mg L^{-1}).

mean *C. triangulifer* LC50 (1956.7 mg L^{-1}) was significantly higher than both *C. dubia* (579.3 mg L^{-1}) and *D. magna* (699.8 mg L^{-1}) by post-Tukey's comparison ($p < 0.001$). The mean *C. dubia* and *D. magna* LC50 do not differ significantly ($p = 0.699$) [KCl acute mortality endpoint sensitivity: *C. triangulifer* < *C. dubia* = *D. magna*].

3.2.2. Chronic tests

Three chronic KCl tests were conducted with each species. Chronic LC50s for *C. triangulifer* could not be estimated, as there was not enough mortality at the highest concentration (2000 mg L^{-1}). An ANOVA of mean chronic LC50s between species showed significant differences (F{2} = 590.183, $p < 0.001$) (Fig. 3). Post-Tukey comparisons show that the *C. triangulifer* mean chronic LC50 (<2000 mg L^{-1}) is significantly higher than the other species ($p < 0.001$). *C. dubia* (638.3 mg L^{-1}) and *D. magna* (673.7 mg L^{-1}) did not differ significantly from each other ($p = 0.727$). [KCl chronic mortality endpoint sensitivity: *C. triangulifer* < *C. dubia* = *D. magna*].

The chronic KCl tests produced a mean IC25 value of 356.2, 338.8, and 615.6 mg L^{-1} for *C. triangulifer*, *C. dubia* and *D. magna* respectively (Fig. 2). An ANOVA between all three species showed no significant difference (F{2} = 3.509, $p = 0.098$) (Fig. 2). However a post-Tukey comparison produced a trend ($p = 0.147$) that *C. triangulifer* had a lower IC25 than *D. magna*, but it was not significant due to the large confidence interval of *C. dubia*. Removing *C. dubia* and performing a pair-wise comparison between *C. triangulifer* and *D. magna* resulted in a significant difference ($p = 0.003$) [KCl growth endpoint sensitivity: *C. triangulifer* {weight} > *D. magna* {weight}].

3.3. $CuSO_4$

3.3.1. Acute tests

Results of three *C. triangulifer* and *C. dubia* acute tests and four *D. magna* acute tests were conducted using $CuSO_4$ are displayed as dose–response curves in Fig. 3S. A between species ANOVA indicated a significant difference in LC50s (F{2} = 73.539, $p = 0.001$) (Fig. 3). Comparisons by post-Tukey analysis showed that *C. triangulifer* had the lowest mean 48 h LC50 (10.7 μg L^{-1}) followed by *C. dubia* with the second lowest LC50 (27.6 μg L^{-1}) ($p = 0.004$). *D. magna* had the highest mean LC50 (54.6 μg L^{-1}) which was significantly greater than *C. triangulifer* and *C. dubia* ($p < 0.001$) [$CuSO_4$ acute mortality endpoint sensitivity: *C. triangulifer* > *C. dubia* > *D. magna*].

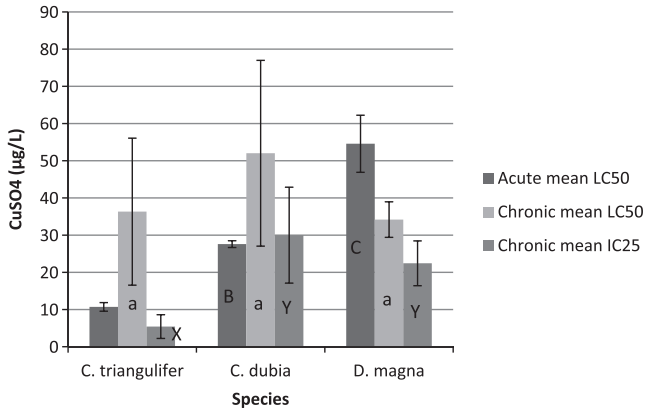

Fig. 3. Results from $CuSO_4$ (μg L^{-1}) acute and chronic tests for all three species. Acute endpoint is depicted as mean LC50 while chronic endpoints include both the mean LC50 and mean IC25 results. ANOVA with post-Tukey results indicate differences between species ($p \leqslant 0.05$). Different letters within a test endpoint indicate a significant difference between mean values. Error bars represent 95% confidence intervals.

3.3.2. Chronic tests

ANOVA results indicate that the mean chronic LC50s did not differ significantly from each other across species (F{2} = 1.565, p = 0.284) (Fig. 3). Mean LC50's were 36.3, 52.0, and 34.2 μg L^{-1} for *C. triangulifer*, *C. dubia*, and *D. magna* respectively. [$CuSO_4$ chronic mortality endpoint sensitivity: *C. triangulifer* = *C. dubia* = *D. magna*].

Once again, in $CuSO_4$ chronic tests, weight was the most sensitive parameter of growth for *C. triangulifer* and was used in between species comparisons. ANOVA comparison of mean IC25s indicated significant differences between species (F{2} = 8.282, $p = 0.011$) (Fig. 3). Post-Tukey analysis indicates the *C. triangulifer* mean IC25 (5.4 μg L^{-1}) is lower than the *C. dubia* mean IC25 (30.0 μg L^{-1}) ($p = 0.010$) [$CuSO_4$ growth/reproduction endpoint sensitivity: *C. triangulifer* {weight} > *C. dubia* {reproduction}]. There is a trend that the *C. triangulifer* mean IC25 is lower than *D. magna* mean IC25 (22.4 μg L^{-1}) ($p = 0.078$), however, due to the large confidence interval of *C. dubia* it is not significant.

Table 1 summarizes the acute and chronic results of the 3 reference toxicants used across all 3-test organisms.

4. Discussion

Overall, this study was able to develop reproducible acute and chronic toxicity procedures for the mayfly *C. triangulifer*. These test procedures were highly reproducible and could be easily followed in different laboratories. *C. triangulifer* response to reference toxicants differed from that of other commonly used ecotoxicogical models. This suggests a role for *C. triangulifer* as a test species that may reflect chemical impacts on under-represented aquatic groups.

4.1. NaCl and KCl

C. triangulifer has proven to be equally or more sensitive than tested model organisms for NaCl and further use of this species in NaCl studies is warranted. *C. triangulifer* is less sensitive than the model organisms with KCl as the toxicant when LC50 is the endpoint, but equally or more sensitive during growth/reproduction studies. In addition, during KCl chronic studies there was a large variation in the number *C. dubia* offspring, which masked any significant difference in mean IC25s between species. The large variation in offspring production was likely due to the 0 to 24-h age range of individuals at the start of the test. To decrease variability in the future, a smaller age window for *C. dubia* young is recommended.

Overall, *C. dubia* and *D. magna* performed as expected in this study based on historical data. This study's acute 48-h LC50 values were compared with endpoints described by Mount et al. (1997). This study's acute average 48 h LC50 for *C. dubia* in KCl was 579 mg L^{-1} which falls very close the 580–670 mg L^{-1} range for 48-h LC50s seen in the earlier study (Mount et al., 1997). The average acute 48-h LC50 of 2504 mg L^{-1} for *C. dubia* in NaCl was slightly above but similar to Mount's 48-h range of 1770–2330 mg L^{-1}. The historical KCl LC50 range at 48 h for *D. magna* is 440–880 mg L^{-1} (Mount et al., 1997). During this study, the average *D. magna* LC50 was within range at 700 mg L^{-1} for acute testing. The *D. magna* 48-h LC50 of 4869 mg L^{-1} also fell within Mount et al. (1997) NaCl range of 3790–5740 mg L^{-1}.

Chronic test results indicated that *C. triangulifer* growth was affected by both NaCl and KCl, and nymphal weight decreased as salt concentration increased. A study by Weaver et al. (accepted for publication) showed that mayfly weight is positively correlated to the number of eggs produced, suggesting that the observed reductions in growth would likely result in reduced fecundity. This

Table 1
Average acute and chronic results for three toxicants for *C. triangulifer*, *C. dubia* and *D. magna*. Values in bold are the lowest values found during this study.

Species	*C. triangulifer*	*C. dubia*	*D. magna*
NaCl acute LC50	**658.7 mg L^{-1}**	2504.3 mg L^{-1}	4868.7 mg L^{-1}
NaCl chronic LC50	**833.1 mg L^{-1}**	1571.3 mg L^{-1}	4312.0 mg L^{-1}
NaCl chronic IC25	**228.8 mg L^{-1}**	988.9 mg L^{-1}	2747.2 mg L^{-1}
KCl acute LC50	1956.7 mg L^{-1}	**579.3 mg L^{-1}**	699.8 mg L^{-1}
KCl chronic LC50	>2000 mg L^{-1}	638.3 mg L^{-1}	673.7 mg L^{-1}
KCl chronic IC25	**356.2 mg L^{-1}**	**338.8 mg L^{-1}**	615.6 mg L^{-1}
$CuSO_4$ acute LC50	**10.7 μg L^{-1}**	27.6 μg L^{-1}	54.6 μg L^{-1}
$CuSO_4$ chronic LC50	**36.3 μg L^{-1}**	52.0 μg L^{-1}	**34.2 μg L^{-1}**
$CuSO_4$ chronic IC25	**5.4 μg L^{-1}**	30.0 μg L^{-1}	22.4 μg L^{-1}
Acute $CuSO_4$ LC50 w/chronic feeding regime	>80.0 μg L^{-1}	>80.0 μg L^{-1}	**68.2 μg L^{-1}**

suggests that if the chronic test was extended to emergence and egg collection, NaCl concentrations greater than 312.5 mg L^{-1} (no observed effect concentration – NOEC) and KCl concentrations greater than 250 mg L^{-1} (NOEC) would cause an effect on fecundity.

4.2. $CuSO_4$

Results from the acute $CuSO_4$ tests show that *C. triangulifer* is significantly more sensitive than *C. dubia* or *D. magna* when using mortality as a 48-h end point. However, there was some discrepancy when examining mortality in chronic tests. The LC50 for *C. triangulifer* averaged 10.7 μg L^{-1} in the acute tests but increased to 36.3 μg L^{-1} in chronic tests. The average LC50 for *C. dubia* increased from 27.6 to 52.0 μg L^{-1} in 7 d tests, while the average LC50 for *D. magna* decreased from 54.6 to 34.2 μg L^{-1} in 4 d tests. Previous work from Suedel et al. (1996) indicate that *C. dubia* and *D. magna* exposed to aqueous copper sulfate reach a mortality threshold at 7 d and 4 d, respectively.

One consideration for the discrepancy between mortality in acute and chronic $CuSO_4$ tests of *C. triangulifer* is variability between mayfly generations. Even though *C. triangulifer* are clonal reproducers, each generation is cultured separately in Lab-line. The conductivity of Lab-line did vary during culturing, due to the conductivity of the incoming tap water. Fluctuations in culture water could account for some variability in sensitivity. In addition, some mayfly eggs were held at 4 °C to delay hatching until use. The duration of delayed hatching did vary and may have caused some variability in results. Further analysis of the effect of delayed hatching on *C. triangulifer* may be beneficial.

An evaluation of acute metal exposures by Brix et al. (2011) indicated that aquatic insects are insensitive to acute metal exposure relative to other aquatic invertebrates. Results from the current study are contradictory to Brix, but Brix does indicate a need for more chronic testing of aquatic insects. While the *C. triangulifer* were sensitive to $CuSO_4$ in acute testing, there was also a growth effect during chronic testing at an even lower concentration, demonstrating the benefit of conducting longer-term exposures.

Other studies using *C. triangulifer* have shown that dietary studies using zinc and selenium are the primary routes of exposure to mayflies for metals and metalloids (Conley et al., 2011; Kim et al., 2012). Our acute exposures used a small amount of diatoms that may or may not have enhanced toxicity but may have also provided a more realistic exposure and ecologically relevant estimate of acute toxicity of salts and metals to mayflies. These dietary studies (Conley et al., 2011; Kim et al., 2012) also support our findings that *C. triangulifer* is an important organism to be included in the derivation of ambient water quality criteria. Using the methods presented in this paper as well as life cycle tests with *C. triangulifer* will improve our understanding of toxic substances effects on aquatic insects.

Further toxicology bioassays using more toxicants should be conducted using these newly validated acute and chronic methods. Toxicology studies that utilize this mayfly species may detect detrimental effects at lower concentrations of aquatic toxicants than if tested with *C. dubia* and *D. magna*. Using a more sensitive model system has the potential to elucidate better water quality information that is more protective of native species. The utilization of this mayfly in future studies will help expand the database of toxicology information on aquatic insects. In addition, it can help to diminish the gap between over-represented cladoceran and under-represented aquatic insects.

Acknowledgements

The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.04.096.

References

Brinkman, S., Johnston, W., 2008. Acute toxicity of aqueous copper, cadmium, and zinc to the mayfly *Rhithrogena hageni*. Arch. Environ. Contam. Toxicol. 54, 466–472.

Brix, K.V., DeForest, D.K., Burger, M., Adams, W.J., 2005. Assessing the relative sensitivity of aquatic organisms to divalent metals and their representation in toxicity datasets compared to natural aquatic communities. Human Ecol. Risk Assess. 11, 1139–1156.

Brix, K.V., DeForest, D.K., Adams, W.J., 2011. The sensitivity of aquatic insects to divalent metals: a comparative analysis of laboratory and field data. Sci. Total Environ. 409, 4187–4197.

Conley, J.M., Funk, D.H., Buchwalter, D.B., 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.

Conley, J.M., Funk, D.H., Cariello, N.J., Buchwalter, D.B., 2011. Food rationing affects dietary selenium bioaccumulation and life cycle performance in the mayfly *Centroptilum triangulifer*. Ecotoxicology 20, 1840–1851.

Diamond, J., Winchester, E., Mackler, D., Gruber, D., 1992. Use of the mayfly *Stenonema modestum* (Heptageniidae) in subacute toxicity assessments. Environ. Toxicol. Chem. 11, 415–425.

Echols, B.S., Currie, R.J., Cherry, D.S., 2010. Preliminary results of laboratory toxicity tests with the mayfly, *Isonychia bicolor* (Ephemeroptera: Isonychiidae) for development as a standard test organism for evaluating streams in the Appalachian coalfields of Virginia and West Virginia. Environ. Monit. Assess. 169 (1–4), 487–500.

Funk, D.H., Jackson, J.K., Sweeney, B.W., 2006. Taxonomy and genetics of the parthenogenetic mayfly *Centroptilum triangulifer* and its sexual sister *Centroptilum alamance* (Ephemeroptera: Baetidae). J. North Am. Benth Soc. 25, 417–429.

Gibbs, K. 1973. The seasonal distribution of *Cleon triangulifer* McDunnough in a pond in eastern Canada. In: 1st International Conference of Ephemeroptera, pp. 39–47.

Hassell, K.L., Kefford, B.J., Nugegoda, D., 2006. Sub-lethal and chronic salinity tolerances of three freshwater insects: *Cloeon* sp. and *Centroptilum* sp.

(Ephemeroptera: Baetidae) and *Chironomus* sp. (Diptera: Chironomidae). J. Exp. Biol. 209, 4024–4032.
Irving, E.C., Baird, D.J., Culp, J.M., 2003. Ecotoxicological responses of the mayfly *Baetis tricaudatus* to dietary and waterborne cadmium: implications for toxicity testing. Environ. Toxicol. 22, 1058–1064.
Kim, K.S., Funk, D.H., Buchwalter, D.B., 2012. Dietary (periphyton) and aqueous Zn bioaccumulation dynamics in the mayfly *Centroptilum triangulifer*. Ecotoxicology 21, 2288–2296.
Lazorchak, J., Smith, M., Haring, H., 2009. Development and validation of a daphnia magna four-day survival and growth test method. Environ. Toxicol. Chem. 28, 1028–1034.
Lowell, R., Culp, J., Wrona, F., 1995. Stimulation of increased short-term growth and development of mayflies by pulp mill effluent. Environ. Toxicol. Chem. 14, 1529–1541.
McDunnough, J., 1931. New species of North American ephemeroptera. Can. Entomologist 63 (4), 82–93.
Mebane, C., Hennessy, D., Dillon, F., 2008. Developing acute-to-chronic toxicity ratios for lead, cadmium, and zinc using rainbow trout, a mayfly and a midge. Water, Air, Soil Pollut. 188, 41–66.
Mount, D., Gulley, D., Hockett, J., Garrison, T., Evans, J., 1997. Statistical models to predict the toxicity of major ions to *Ceriodaphnia dubia*, *Daphnia magna* and *Pimephales promelas* (Fathead Minnow). Environ. Toxicol. Chem. 16, 2009–2019.
Standley, L.J., Sweeney, B.W., Funk, D.H., 1994. Maternal transfer of chlordane and its metabolites to the eggs of a stream mayfly *Centroptilum triangulifer*. Environ. Sci. Technol. 28, 2105–2111.
Suedel, B., Deaver, E., Rodgers, J., 1996. Experimental factors that may affect toxicity of aqueous and sediment-bound copper to freshwater organisms. Environ. Contam. Toxicol. 30, 40–46.
Sweeney, B.W., Vannote, R.L., 1984. Influence of food quality and temperature on life history characteristics of the parthenogenetic mayfly, *Cloeon triangulifer*. Freshwater Biol. 14, 621–630.
Sweeney, B.W., Funk, D.H., Standley, L.J., 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.
United States Environmental Protection Agency, 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 5th ed. EPA-821-R-02-012. U.S. Environmental Protection Agency, Washington, DC, USA.
United States Environmental Protection Agency, 2002b. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013. U.S. Environmental Protection Agency, Washington, DC, USA.
Weaver, P., Lazorchak, J., Struewing, K., Decelles, S. J., Funk, D.H., Buckwalter, D.B., Johnson, B.R., accepted for publication. Part 1: Laboratory culture of *Centroptilum triangulifer* (Ephemeroptera: BAETIDAE) using a standardized diet of three diatoms. Chemosphere.
Xie, L., Buchwalter, D.B., 2011. Cadmium exposure route affects antioxidant responses in the mayfly *Centroptilum triangulifer*. Aquat. Toxicol. 105, 199–205.
Xie, L., Funk, D.H., Buchwalter, D.B., 2010. Trophic transfer of cd from natural periphyton to the grazing mayfly *Centroptilum triangulifer* in a life cycle test. Environ. Pollut. 158, 272–277.