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A New Toxicity Test Using the Freshwater Copepod *Cyclops vernalis*

Emma M. Marus[1] · James R. Elphick[1] · Howard C. Bailey[1]

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Abstract The cladocerans *Ceriodaphnia dubia* and *Daphnia magna* are widely used in environmental toxicity testing and the test methodologies for these species are well developed. However, copepods are a much more abundant contributor to zooplankton in many lakes, but they are not routinely used in toxicity tests. Therefore, we propose toxicity test methods for the freshwater copepod, *Cyclops vernalis* assessing effects on its survival and growth. A case study is presented in which the proposed test was performed with a range of concentrations of total dissolved solids (TDS) and used as part of a test battery to develop a site-specific water quality objective. *C. vernalis* was less sensitive to TDS compared to *D. magna* and *C. dubia*, but similarly sensitive to an alga, a diatom, a rotifer, a chironomid, and two fish species. No adverse effects were observed on survival or growth of *C. vernalis* at TDS concentrations up to 1500 mg/L.

Keywords Copepod · Freshwater · Toxicity · *Cyclops vernalis* · Total dissolved solids

The Copepoda comprise a highly diverse and widely distributed taxonomic subclass. Although more diverse in marine ecosystems, approximately 2814 freshwater species have been identified, occurring in the vast majority of freshwater ecosystems (Boxshall and Defaye 2008). They are significant contributors to freshwater zooplankton communities, particularly in oligotrophic systems where their relative contribution to abundance of crustaceans increases relative to cladocerans (Carney 1990). For example, Swadling et al. (2000) studied 30 lakes in the Yukon and Northwest Territories (NWT), Canada, and reported that all the lakes contained at least one species of copepod, with *Cyclops* spp. being among the most frequently encountered species. Conversely, these authors reported that alpine tundra lakes contained a general paucity of cladocerans.

Toxicity test methods for species of zooplankton are desirable for environmental investigations as a result of their high degree of sensitivity to contaminants and relatively short life cycles, which make them amenable to laboratory testing. However, despite the importance of copepods to freshwater ecosystems, relatively little information is available on their sensitivity to toxicants and laboratory methods have not been formalized (Kulkarni et al. 2013). In contrast, cladocerans, such as *Ceriodaphnia dubia* and *Daphnia magna,* have been widely used in environmental toxicity testing programs to evaluate chemicals and effluents for potential risks to zooplankton and other invertebrates (Environment Canada 2007; USEPA 2002; ASTM 2004).

Toxicity tests have been conducted with marine copepods, but not routinely. Bengtsson (1978) reported test methods for acute exposures to the marine harpacticoid copepod *Nitocra spinipes*. Chronic exposures have been performed using a number of species, including *Tigriopus japonicus* (Kwok et al. 2009; Dahl et al. 2009), *Tisbe battaglii* (Hutchinson et al. 1999; Diz et al. 2009), *Tisbe biminiensis* (Lavorante et al. 2013), *Acartia tonsa* (Kusk 1997) and *Labidocera aestiva* (Cohen et al. 2014). Finally, the OECD has recently developed methods for full life cycle tests of marine copepods, including *Amphiascus tenuiremis, N. spinipes*, and *A. tonsa* (OECD 2007).

For freshwater copepods, Baudouin and Scoppa (1974) evaluated *Cyclops abyssorum prealpinus* for acute

✉ James R. Elphick
james@nautilusenvironmental.com

[1] Nautilus Environmental, 8664 Commerce Court, Burnaby, BC V5A 4N7, Canada

sensitivity to a range of metals and concluded they were generally less sensitive in short-term exposures than *Daphnia hyalina*. Effects of metal mixtures on biomass of copepods have been evaluated using mixed cultures of cyclopoid copepods (Borgmann 1980). Brown et al. (2005) developed toxicity methods for the harpacticoid copepod *Bryocamptus zschokkei* using a microplate in which copepods were held individually for 20 days, at which point most copepods had achieved adulthood. Males and females were then paired together and a reproductive endpoint was assessed by the presence of egg sacs and nauplii at the end of the test. Burton et al. (2002) evaluated the time it took for the same species to develop from nauplii to copepodite stages. The cyclopoid copepod *Mesocyclops leuckarti* has recently been identified as a potentially useful species for conducting laboratory toxicity tests (Kulkarni et al. 2013).

As part of toxicity testing required to develop a site-specific water quality objective (SSWQO) for total dissolved solids (TDS) discharge in effluent to Snap Lake (NWT, Canada) by the Snap Lake Diamond Mine, we developed two different toxicity test methods using the cyclopoid copepod *Cyclops vernalis* and applied them to the specific blend of ions comprising Snap Lake TDS. The Cyclopoidae are the most numerous family of freshwater copepods (Boxshall and Defaye 2008), and *Cyclops* spp. are found in Snap Lake. Both test methods involved a 20-day exposure and measured survival and growth; one method also measured reproduction.

Methods and Materials

Copepods were obtained from Boreal Science (St. Catherines, ON, Canada) in January 2014, and identified as *Cyclops vernalis* by a qualified taxonomist. Copepod cultures were established using approximately 30 individuals in 1-L glass beakers in culture water, which was prepared by reconstituting deionized water with reagent grade salts to achieve moderately hard water (80–100 mg/L as $CaCO_3$) according to the recipe specified in USEPA (2002). The cultures were maintained under a 16:8 h light–dark photoperiod in a temperature-controlled room at 22 ± 2°C. The culture water was gently aerated (one to two bubbles per second) and partially replaced weekly. Every 2–3 weeks, new copepod cultures were initiated by transferring 30 gravid females from the existing culture to new culture containers in order to thin the density of organisms and prevent build-up of algae on the sides of the culture vessel.

The cultures were fed three times per week with cells of a green alga, *Pseudokirchneriella subcapitata* at a rate of approximately 5×10^5 cells/mL, together with digested yeast, cerophyll, and trout chow (YCT) at a rate of 30 mg/L solids. Based on the observed reproductive success of the cultures, this mixture was considered a suitable food source for both culturing and testing purposes. However, it was unclear whether the copepods were also feeding on their own napulii since adult cyclopoid copepods have been described as carnivorous feeders (Nandini and Sarma 2007; Santer 1998). The potential for carnivorous behavior was minimized through provision of a suitable food source and regular thinning of the cultures. Once the culture was stable and reproduction was consistently occurring, testing was initiated with nauplii of <0.2 mm in size, obtained by gently filtering a subsample of the culture through a 0.2 mm Nitex screen.

Testing was conducted using a range of TDS concentrations whose composition was based on the concentrations of major ions present in Snap Lake, dominated by chloride and calcium. Water prepared to simulate Snap Lake water (i.e., synthetic lake water) was prepared by dissolving reagent-grade sodium, potassium, calcium, and magnesium salts (219.8 mg/L NaCl, 26.3 mg/L KCl, 35.2 mg/L $MgCl_2 \cdot 6H_2O$, 161.3 mg/L $MgSO_4$, 1106.1 mg/L $CaCl_2 \cdot 2H_2O$, and 185.3 mg/L $NaHCO_3$) in reverse osmosis-treated deionized water. The nominal TDS concentration of the synthetic lake water was 1500 mg/L, with ion ratios representative of Snap Lake.

Dilutions of the synthetic lake water were prepared using deionized water with a 0.67 X dilution factor to achieve a nominal concentration series of: 1500; 1000; 667; 444; and, 296 mg TDS/L. Test solutions were analyzed for ionic composition to determine the actual concentrations of the major ions, which were then used to calculate actual TDS concentrations by summing the ions. One batch of the highest nominal concentration of the TDS solutions (i.e., 1500 mg/L) was prepared prior to test initiation and was used for preparing dilutions throughout the tests.

A reference toxicant test was conducted using cadmium. Cadmium was chosen because invertebrates are sensitive to this metal, and the toxicity testing laboratory had data for *C. dubia* and *D. magna* with which to compare the results. The test concentrations (2, 4, 8, 16, and 32 µg Cd/L) were prepared using a stock solution made by dissolving reagent-grade $CdCl_2$ in deionized water. Test concentrations were prepared by mixing the required amount of stock solution with moderately hard water, the same medium used for culturing the copepods. The test solutions were analyzed for total Cd using ICP-MS to determine actual concentrations, which were then used for statistical analyses.

Two test methods, identified as Method A and Method B, were used to evaluate survival and growth of *C. vernalis* in the TDS solutions. For the cadmium exposure, only one toxicity test was conducted and followed conditions

described for Method B. The primary difference between the methods was that Method A involved exposure of ten copepods in each replicate, which provided the opportunity for reproduction to occur, whereas Method B involved exposure of individually isolated organisms. The latter method prevented an assessment of reproduction in the test; however, it removed the potentially confounding influence of cannibalism.

The experimental design of Method A consisted of four replicates of 375 mL jars containing approximately 300 mL of test solution for each concentration. Each jar contained a screened-tube comprised of 25 µm Nitex screen sandwiched between two pieces of 4 cm inside diameter Plexiglass cylinder (Fig. 1). In each replicate, ten nauplii (less than 0.2 mm) were placed in the screen tube, above the Nitex screen. This apparatus was designed to enable water changes to occur without disturbing or losing the test organisms; the test solution could be drawn down by syphoning the water from the glass jar, outside of the screen tube. Similar to culturing conditions, test organisms were fed three times per week and maintained at 22 ± 2°C with a 16:8 h light–dark photoperiod. Water changes occurred twice a week, at which time pH, dissolved oxygen, and conductivity of old and new solutions were measured. Temperature was recorded daily.

The test organisms were observed daily, at which time mortalities were recorded and removed. Mortalities were defined as organisms showing no heartbeat when observed under a dissecting microscope. In cases where an egg sac was observed on a female copepod, the female was removed from the test container using a glass pipette and isolated in test solution in a 20 mL glass test tube so that number of nauplii produced could be assessed; gravid adults were monitored daily, and nauplii were typically released from the egg sac within 2–3 days.

Method B was carried out following a similar approach to the standard methods used to conduct chronic toxicity tests using the cladoceran *C. dubia* (Environment Canada 2007; USEPA 2002). Testing was conducted in 20-mL glass test tubes holding 15 mL of test solution, with a single nauplius (less than 0.2 mm) placed in each test tube at test initiation. Testing was conducted with ten replicates for each concentration. Feeding, light, temperature conditions and solution renewals were the same as presented for Method A. Test solutions were renewed by carefully removing the copepod with a glass pipette from the test tube, rinsing the test tube with deionized water, adding 15 mL of fresh solution into the clean test tube, and then replacing the organism back into the test tube.

The tests were terminated after 20 days, at which time the final survival was recorded and the adult copepods were preserved in ethanol. Length, from tip of the rostrum to the end of the pleopods, was measured using a dissecting microscope with a calibrated ocular micrometer. The copepods were too small for weight to be a discriminatory measure. Since male copepods of this species are smaller than females, the gender of the individuals was recorded so that growth of male and female copepods could be evaluated separately.

The data were analyzed statistically using CETIS (Tidepool Scientific Software 2013) and following the recommendations of Environment Canada (2005). Point estimates for survival were calculated using maximum likelihood estimation using the best fit of either probit, logistic or Gompertz models, and effects on growth were calculated using linear interpolation (using log transformed concentration data). Nonlinear regression is a preferred approach for analyzing continuous data such as those associated with a growth endpoint (Environment Canada 2005); however, these models did not fit the data in this case.

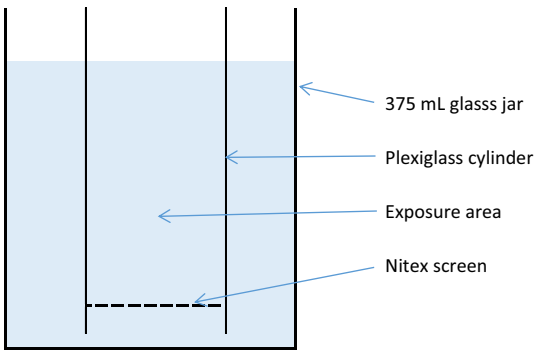

Fig. 1 Test apparatus for exposures conducted using *Cyclops vernalis* according to Method A

Results and Discussion

Water quality measurements indicated that dissolved oxygen (>70 % saturation) and pH (ranged from 6.8 to 8.2) remained within suitable ranges throughout exposure. Results of the tests using TDS and cadmium are shown in Tables 1 and 2, respectively. Control survival ranged from 70 % to 80 % in the three tests; this rate of survival is not ideal, but is consistent with the ASTM (2004) requirements for a similar duration test using *D. magna* (i.e., ≥70 % survival over 21 days). Thus, this rate of survival reflects a reasonable control performance metric for this test. We would expect the control survival to improve with additional experience in handling the organisms and with the test procedures in general to be ≥80 % survival, which is more commonly associated with chronic toxicity test methods.

Table 1 Survival and growth of *Cyclops vernalis* exposed to total dissolved solids (TDS) for 20 days using two methods of exposure

Concentration (mg TDS/L)		Survival (%) (mean ± SD)	Female length (mm) (mean ± SD [n])	Male length (mm) (mean ± SD [n])
Nominal	Measured			
Method A				
Control		78 ± 5	1.00 ± 0.06 [n = 21]	0.72 ± 0.09 [n = 10]
296	295	63 ± 17	0.97 ± 0.10 [n = 20]	0.74 ± 0.15 [n = 6]
444	441	73 ± 10	1.14 ± 0.06 [n = 19]	0.91 ± 0.09 [n = 10]
667	666	70 ± 10	1.24 ± 0.06 [n = 15]	0.93 ± 0.05 [n = 6]
1000	1008	60 ± 8	1.13 ± 0.07 [n = 17]	0.94 ± 0.07 [n = 7]
1500	1508	60 ± 8	0.94 ± 0.04 [n = 18]	0.71 ± 0.06 [n = 6]
Test endpoint				
LC50 (mg/L)		>1508	–	–
IC20 (mg/L)		–	>1508	>1508
Method B				
Control		70	0.69 ± 0.14 [n = 6]	0.52 [n = 1]
296	278	80	0.84 ± 0.23 [n = 4]	0.66 ± 0.11[n = 4]
444	430	80	0.69 ± 0.12 [n = 5]	0.48 ± 0.03 [n = 3]
667	645	70	0.79 ± 0.15 [n = 6]	0.48 [n = 1]
1000	978	70	0.74 ± 0.15 [n = 6]	0.50 [n = 1]
1500	1508	80	0.65 ± 0.17 [n = 7]	0.70 [n = 1]
Test endpoint				
LC50 (mg/L)		>1508	–	–
IC20 (mg/L)		–	>1508	>1508

[n] reflects the total number of measured organisms, which were divided across four replicates in Method A and exposed individually in Method B

Table 2 Survival and growth of *Cyclops vernalis* exposed to cadmium for 20 days

Concentration (μg Cd/L)		Survival (%) (mean ± SD)	Female length (mm) (mean ± SD [n])	Male length (mm) (mean ± SD [n])
Nominal	Measured			
Control		80	0.71 ± 0.04 [n = 3]	0.76 ± 0.18 [n = 5]
2	1.8	70	0.49 [n = 1]	0.60 ± 0.13 [n = 6]
4	3.7	70	0.69 ± 0.20 [n = 4]	0.63 ± 0.12 [n = 3]
8	7.4	40	0.54 ± 0.04 [n = 3]	0.82 [n = 1]
16	14.8	0	–	–
32	29.6	0	–	–
Test endpoint				
LC50 (95 % CL)		7.1 (5.2–9.5)	–	–
IC20 (95 % CL)		–	6.5 (not calculable)	>7.4

Survival in the various test solutions ranged from 60 % to 78 % using Method A, and from 70 % to 80 % using Method B (Table 1). There was no discernable relationship between TDS concentration and survival, although the lowest rate of survival (60 %) was observed in the highest two test concentrations using Method A. No mortalities were observed until day 13 of the exposure using Method A, with occasional mortalities observed in all replicates and test concentrations over the following 7 days. Based on the pattern of mortalities (i.e., that mortalities occurred primarily during the latter part of the test), it is possible that cannibalism contributed to mortalities that were observed during the last week of the test with Method A (Nandini and Sarma 2007; Santer 1998). Rates of survival were somewhat better with Method B; again, there was no evidence of a dose-related effect on survival. In contrast to the results for Method A, the mortalities that occurred using Method B were observed randomly throughout the 20-day exposure, rather than being limited to the final week; the potentially confounding influence of cannibalism was removed in this method by isolating individual copepods in the test replicates.

Growth of both male and female copepods was generally similar to the control, or somewhat enhanced in the

TDS solutions in both tests; the size of the copepods was similar in the highest test concentration compared with the controls. There was no adverse effect on growth of the copepods associated with exposures of up to 1508 mg TDS/L.

The data for length exhibited a high degree of consistency between replicates using Method A, with a coefficient of variation (CV) of approximately 6 % for females and 10 % for males. The patterns of growth were similar for males and females, with enhanced growth in the intermediate concentrations of TDS relative to the control. Males were consistently smaller than females by 20 %–25 %. Method B exhibited a larger CV for female growth, which is not surprising since there were only ten organisms exposed per concentration (separately in ten replicates) in this test, compared with 40 organisms in Method A (using four replicates of ten organisms). The number of males in the Method B exposure was small, with four of the concentrations having only a single male, preventing an assessment of the CV for male growth. Interestingly, the difference in size between male and female copepods was smaller in Method B, where the organisms were housed individually, and the females were smaller than they were using Method A, suggesting that the presence of other copepods during the test might influence growth. Competition has been shown to affect organism performance in tests using other zooplankton (Liess and Foit 2010), and Method B removes this potentially confounding factor, while also removing the potential for cannibalism.

Despite the higher variability, the minimum significant difference (MSD) associated with female growth using Method B was 23.2 %, compared with 6.9 % for Method A, which indicates that both methods had the ability to detect a 20 %–25 % change from the control with a reasonable degree of confidence. The MSD is primarily used in association with hypothesis tests, however, it also provides a useful measure of confidence in calculation of point estimates (deBruyn and Elphick 2013). Although both of these test methods produced growth results that had a reasonable sensitivity to detect adverse effects, future use of Method B would benefit from increased replication to further improve statistical power and to provide additional data on differences between males and females.

The results of Method A indicate that a coefficient of variation of 10 % or less is a reasonable expectation for the mean and standard deviation growth of this species. Based on a desire to detect a 20 % deviation from the control as being statistically significant, power analysis indicated that sample sizes of six and seven should be sufficient to achieve 80 % and 90 % power, respectively. In order to account for differences in male and female growth, a total of twenty organisms per concentration is considered desirable for future application of this test using Method B.

Reproduction was observed using Method A, indicating that the copepods had reached maturity in the 20 day exposure. However, only six female copepods produced nauplii during the test: three in control replicates; and one in each of the 295, 441, and 1008 mg TDS/L solutions; brood sizes for these six females ranged from 8 to 16 nauplii. In addition, three females in a control replicate, and one in the each of the 441, 666, and 1508 mg TDS/L solutions were holding eggs at the end of the test. The rate of reproduction was too low and variable to evaluate any differences between concentrations. Thus, growth was the most useful sublethal endpoint from this test.

The results of the reference toxicant test using cadmium are provided in Table 2. The LC50 for cadmium was 7.1 µg/L, which reflects a similar sensitivity to results from toxicity tests using *C. dubia* and *D. magna* performed under similar conditions of water hardness, which produced LC50 values of 9.5 and 9.9 µg Cd/L, respectively (Nautilus Environmental, unpublished data). Growth was not substantially more sensitive to cadmium than survival; the IC20 for growth was 6.5 and >7.4 µg Cd/L for female and male copepods, respectively. This species is less sensitive to cadmium than long-term exposures using *Hyalella azteca*, which was identified as the most sensitive species to cadmium in a recent water quality guideline derivation, having exhibited effects on growth at concentrations as low as 0.5 µg Cd/L (BCMoE 2015).

The methods presented here demonstrate a sensitive and feasible approach for conducting toxicity tests with *C. vernalis*. The methods have particular relevance as an alternative to tests using cladocerans to assess impacts on environments where copepods predominate in the zooplankton, as they do at Snap Lake. The data presented in these tests indicate no evidence of adverse effects on *C. vernalis* survival or growth relative to the control in concentrations of up to 1508 mg TDS/L, based on a site-specific mixture of ions. As previously noted, this testing was part of a battery of tests designed to determine a site-specific water quality objective for TDS in Snap Lake. On the basis of this testing, *C. vernalis* was less sensitive to TDS compared to the cladocerans *D. magna* and *C. dubia*, but responded similarly to an alga, a diatom, a rotifer, a chironomid, and two fish species (Chapman and McPherson 2015). However, sensitivity of survival of *C. vernalis* exposed to cadmium was similar to that observed with *C. dubia* and *D. magna*, indicating that they are a sensitive species.

For future testing with this species, Method B is considered preferable to Method A, since potentially confounding influences of competition and cannibalism are removed by isolating the copepods, and reproduction in Method A was considered to be too variable to provide a sensitive and reliable test endpoint.

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