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Assessing the Performance of a Bdelloid Rotifer *Philodina acuticornis odiosa* Acute Toxicity Assay

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Abstract A bioassay using the bdelloid rotifer, *Philodina acuticornis odiosa*, was evaluated for use as a standard test method for direct toxicity assessment testing in the Australasian region. *Philodina acuticornis odiosa* was found to be relatively tolerant to phenol (24 h LC50, 142 mg/L). The mortality endpoint was both reliable and repeatable (the coefficients of variation for mortality at the 24 h LC50 concentration ranged from 11%–24% (n = 8)), sufficiently low to warrant further testing with a range of reference toxicants, so that this organism can be included for use as a regulatory test in Australasia.

Keywords Bdelloid rotifer · Phenol · Toxicity testing · Australia

Direct Toxicity Assessment (DTA) testing is used in the regulatory framework of many countries, but is still in its infancy in Australia compared to the United States of America, United Kingdom, Ireland, Sweden and Denmark (ANZECC and ARMCANZ 2000), and, while some countries have developed standard acute and chronic DTA testing procedures (e.g. the US EPA currently has 10 freshwater and marine test species listed), the development of protocols in Australia has generally been on a regional or site-specific basis (ANZECC and ARMCANZ 2000).

Aquatic invertebrates are widely used in ecotoxological studies, because they are relatively easy to maintain under test conditions, their use is not (as yet) restricted by ethical concerns, and as a group they are generally more sensitive to a range of pollutants (including many pesticides) than vertebrates or plants. Many, if not most, toxicological experiments examining the effects of toxicants on aquatic invertebrates have used cladocerans to represent zooplankton species in their target aquatic environments. However, in Australian inland freshwaters cladocerans are often present at relatively low abundances in comparison to other invertebrate species, such as rotifers and copepods (Ellis 2006). For example a recent survey of zooplankton communities in Lake Hawthorn and the Cardross Lakes in north-western Victoria (Australia) found that rotifers and copepods were the most abundant species (Ellis 2006). This suggests that the use of cladoceran data may have limited usefulness in assessing ecological risks of contamination in many regions of inland Australia, e.g. such as northern Victoria.

The Bdelloidea represent an important group of invertebrates ecologically, with 146 species having been recorded in Australia and New Zealand, of which 15% are endemic (Ricci 1987), warranting examination of their use in toxicity tests. However, the Bdelloidea have been little used in toxicity testing, perhaps because, unlike the Monogononta, bdelloid rotifers do not produce resting eggs which can be stored by laboratories, and reproduce exclusively asexually, with the resultant need for laboratories to maintain actively-growing cultures, and identify and transfer same-aged individuals into test vessels. Currently, there are six major freshwater invertebrate toxicity bioassays suggested for use in Australia and New Zealand (ANZECC and ARMCANZ

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2000), none of which uses a freshwater rotifer. Consequently, this reconnaissance study focussed on (1) the development of the bdelloid rotifer assay, (2) its consistency and (3) the relative sensitivity of *Philodina acuticornis odiosa* to a common reference toxicant, namely phenol, to assess its usefulness in light of all its assets and liabilities, including ecological relevance, sensitivity, protocol development, type of endpoint and variability.

Materials and Methods

A commercially available algae start-up culture (*Chlorella* sp.; Southern Biological Melbourne, Australia) was cultured in Tamiya's algal medium (Vasser 1989) on an ad hoc basis, i.e. whenever algae were required to feed the rotifer culture. Tamiya's algal medium was prepared in glass flasks using deionised water, and was autoclaved at 121°C for 20 min before use. The algae were cultured on a light-table with continuous illumination of 1,000 lux and with continuous aeration for about 7 days, or when the culture achieved a deep-green colour.

A *P. acuticornis odiosa* rotifer start-up culture was obtained from a commercial supplier (Southern Biological, Melbourne, Australia). Initially, rotifers were fed white rice grains, but once a culture of *Chlorella* sp. algae was established, rotifers were fed an algal mixture containing approximately 5×10^5 cells/mL. Rotifers were sub-cultured by renewing food and dilution water every 76 h. Dilution water was prepared with distilled water according to ASTM (2004), and consisted of a mixture of $NaHCO_3$, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KCl at 96, 38.7, 60 and 4 mg/L, respectively. The dilution water was continuously aerated and discarded every 7 days. Temperature (°C), water hardness (mg/L of $CaCO_3$), pH, conductivity (μS/cm), and dissolved oxygen (% saturation) was measured once for every freshly-made batch of dilution water. If necessary, the pH of the dilution water was adjusted to 7.5 with 10% HCl or 10% NaOH before use.

Tests were conducted using juvenile rotifers. The life span of a typical *Philodina* rotifer is about 20 days. A juvenile, or non-egg-laying, rotifer is less than about 5 days old. There is a clear distinction in size between adults and juvenile rotifers, albeit only for a limited period of time. Thus rotifers that appeared much smaller in size in comparison to full-fledged adults were collected and used directly in the testing program. Although this protocol complies with the ASTM's suggestion that test organisms should be as young as possible (ASTM 1996), it does not guarantee that the organisms are neonates less than 24 h old. Hence the generic term, juvenile.

Phenol (99+% purity) standards (Sigma Aldrich Pty. Ltd, Castle Hill, Australia) were used in the toxicity testing. Stock standard solutions of phenol were prepared by dissolving the required amount of chemical in 1 mL of acetone (AR grade, Mallinckrodt Chemicals). Solutions were then made up to volume with dilution water. Stock solutions were serially diluted with dilution water to achieve the required test concentrations. The highest concentration of acetone present in a test solution was 0.00001% v/v, and a solvent vehicle control incorporating this amount of acetone was included in test regimes.

At the start of each test, the standard solutions used in the tests were measured for temperature, hardness (mg/L of $CaCO_3$), pH, and electrical conductivity (EC) using an alcohol thermometer, a YSI 9100 photometer (YSI Inc., Yellow Springs, USA) and hardicol hardness tablets (Aquasonic Pty Ltd, Wauchope, Australia), a combined pH and DO meter (TPS Ionode WP-81, TPS Pty Ltd, Springwood, Australia), and a conductivity meter (Hanna Instruments HI 9635, Hanna Instruments, Portugal,), respectively. Dissolved oxygen (% saturation) was measured only before experiments, as per the recommendations of the ASTM guidelines (ASTM 2004), using a combined pH and DO meter (TPS Ionode WP-81, TPS Pty Ltd, Springwood, Australia). Rotifers can survive in low dissolved oxygen conditions, therefore this parameter was not considered critical for the tests undertaken.

All tests were carried out according to the ASTM standard guidelines for acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians (ASTM 1996), with modifications made based on the latest methods available for rotifers (ASTM 2004). A light intensity of 600 lux, a light cycle of 16 h light and 8 h dark, and a temperature of 21.3 ± 0.16°C to simulate a mild summer's day was used in all tests, which were conducted in 24-multiwell tissue culture plates with a test well volume of 1 mL. Juvenile rotifers were transferred from their culture dishes to each test well using a glass micropipette so that there were about 10 animals in each well, with the exact number of rotifers transferred into each well recorded at the start of the test. Rotifers were transferred under a microscope (WILD HEERBRUGG M3Z dissecting microscope, used at 40× magnification). Dilution water transferred with the rotifers was drained from each well with the use of a glass micropipette, and test solutions were added to the wells. After 24 h, each test well was examined under a microscope and the number of dead rotifers in each test well counted and the percentage mortality determined. After a 24-h exposure period, percent mortality was recorded for each well. Rotifers were considered dead if they were not moving and observation confirmed by the clearing of internal tissues. Tests were accepted if there was at least 90% survival in the controls.

A definitive toxicity test was undertaken using 100, 150, 200, 250 and 300 mg/L phenol. Testing also included a

negative control (dilution water only, 0 mg/L phenol) and a solvent vehicle control (dilution containing 0.00001% v/v acetone). Each treatment was replicated four times. Rotifers were exposed to nominal concentrations of the test chemical.

From the definitive test described above, the LC50 value was calculated. Thereafter, rotifers were exposed to this calculated LC50 concentration on a further eight occasions over a 4 month period. At the time of each consistency test, this treatment was replicated 8 times, and again rotifers were exposed to nominal concentrations of test chemical.

Data from the toxicity testing was analysed using Genstat Version 9.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2006). Effective lethal doses from the definitive phenol acute toxicity tests were determined by fitting a regression model using the probit method. The probit analysis was conducted on the log10 of the chemical concentrations that were then back transformed to state LC50 values as a concentration in water (i.e. mg/L). One-way analysis of variance (ANOVA) on the log10 of the mortality responses was used to determine whether the test was temporally consistent, with significance tested at the 95% confidence level.

Results and Discussion

Philodina rotifers have been vastly found in a variety of different habitats throughout Australia, including acid waters, mosses, and the open waters of stock dams, lakes, and rivers (Koste and Shiel 1986). Consequently, it was not possible to define test water quality based on a specific set of physico-chemical parameters, and so ASTM water was used instead. The purpose of using ASTM water was to provide *Philodina* rotifers with the optimum freshwater environment, i.e. with everything they needed for survival, ensuring that mortality could not be influenced by anything but the test chemical, and an environment that could be reproduced in most ecotoxicology laboratories. The solvent vehicle control was conducted for the purpose of rejecting or accepting the test, i.e. if mortality in solvent vehicle control exceeded 10%, the test was rejected. This, however, never happened.

Average mortality in the negative control in the direct toxicity tests was ~2% (Table 1), and therefore the tests were considered acceptable (ASTM 2004). Accordingly, the 24 h LC50 was calculated to be 142 mg/L phenol (95% confidence intervals (95% CI), 125–157 mg/L), which suggests the rotifers used in this study are reasonably tolerant to phenol. This is consistent with Calleja et al. (1994) who reported a 24 LC50 of 150 mg/L for the freshwater rotifer, *Brachionus calyciflorus*, but one quarter of that reported for the freshwater rotifer *B. rubens* (24 h LC50, 600 mg/L; Halbach et al. 1983), and approximately one third of that determined for the marine rotifer *B. plicatilis* (24 h LC50, 400 mg/L; Snell et al. 1991). Data on the toxicity of phenol to other freshwater zooplankton species is relatively limited, however *P. acuticornis odiosa* is also an order of magnitude less sensitive to phenol than zooplankton such as the opossum shrimp, *Americamysis bahia,* and the cladoceran, *Daphnia magna* (24 h LC50 14 and 29 mg/L, respectively; Table 2).

Table 1 Summary of mortality observed when the freshwater rotifer *P. acuticornis odiosa* was exposed to phenol

Treatment (mg/L phenol)	Mortality (%) (n = 4)		
	Mean	Range	CV (%)
0	1.6	0–6	200
100	35.2	9–60	61
150	42.4	25–59	35
200	86.5	80–93	6
250	92.5	77–93	12
300	93.9	89–100	5

Within laboratory variability (CV) in biological test methods is often 20%–40% (Bruno and Eklund 2003). The coefficients of variation (CV) for this test's 24 h LC50 were ~15%, and there was no statistically significant difference between observed mortality between any of the consistency tests ($p > 0.05$), i.e. this *P. acuticornis odiosa* bioassay generated reproducible estimates of phenol toxicity. The test also proved to be very temporally accurate, i.e. when the organisms were exposed to the calculated LC50 concentration (142 mg/L) on 8 separate occasions, the mean percentage mortality on each occasion was within 10% of the expected value (Fig. 1).

The use of resting eggs in acute toxicity tests has been the biggest factor by far in facilitating testing with monogonont rotifers. Unfortunately, rotifer resting eggs are not readily available for purchase in Australia, and strict quarantine regulations have restricted importation of kits using resting eggs. This has both limited studies on native Australian monogonont rotifer species, and the use of resting egg-based kits in testing regimes. The bdelloid test described herein was in part developed to overcome these restrictions, although in and of itself it has some limitations. For instance, rotifers were selected according to their size compared to a full-fledged adult. This means that the rotifers in the test can be anywhere from newly-hatched to 5 days of age, but are more likely to be the former because a *Philodina* rotifer reaches its adult life stage (and therefore its egg-laying stage) at about 5 days of age. At this point in time, the rotifers have reached their maximum length. Consequently, the test is reproducible, as long as a laboratory has full-fledged adult *Philodina* to compare test

Table 2 Relative sensitivity of the freshwater rotifer *P. acuticornis odiosa* to phenol

Organism		24 h LC50 (mg/L)	Reference
Americamysis bahia	Opossum shrimp	14	Buikema et al. 1981
Daphnia magna	Water flea	29	LeBlanc 1980
Acartia clausi	Calanoid copepod	32	Buttino 1994
Palaemonetes pugio	Grass shrimp	53	Tatem et al. 1978
Gammarus pulex	Scud	112	Stephenson 1983
P. acuticornis odiosa	Bdelloid rotifer	142	This study
Brachionus calyciflorus	Rotifer	150	Calleja et al. 1994
Artemia salina	Brine shrimp	160	Price et al. 1974
Tisbe battagliai	Harpacticoid copepod	172	Smith et al. 1994
Brachionus plicatilis	Rotifer	400	Snell et al. 1991
Brachionus rubens	Rotifer	600	Halbach et al. 1983
Tetrahymena pyriformis	Ciliate	600	Roberts and Berk 1990

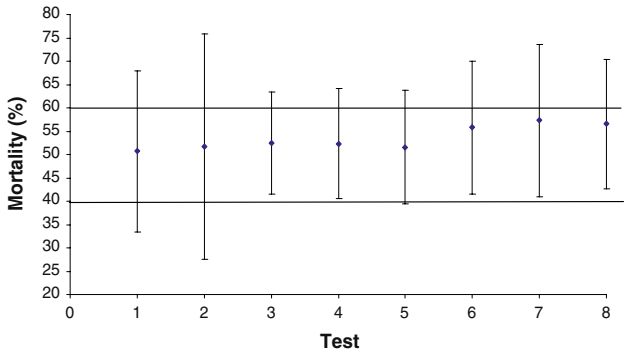

Fig. 1 Summary of response of *P. acuticornis odiosa* acute toxicity assay relative to expected performance at LC50 concentration. Error bars represent the coefficient of variation (%) in test data. Solid horizontal lines represent 50% response ± 10%, respectively

animals against when selecting juveniles for testing, i.e. anything smaller than the typical full-fledged adult is considered a juvenile. However, laboratories will need to maintain a viable stock culture of both rotifers and algae in order to have a healthy culture and reliable supply of juvenile rotifers. Moreover, there are some difficulties associated with catching and transferring juveniles accurately. That said, this preliminary study has indicated that the degree of variability in the *P. acuticornis odiosa* acute toxicity assay is sufficiently low to warrant further investigation of its use, and in particular further testing with a range of reference toxicants, so that this organism can be included for use as a regulatory test in Australasia.

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