DEVELOPMENT OF AN ACUTE TOXICITY TEST WITH THE MARINE COPEPOD ACARTIA SINJIENSIS

Amanda Rose, Ann-Marie Carruthers, Jenny Stauber, Richard Lim and Stephen Blockwell

INTRODUCTION

Direct toxicity assessment is an important component of the Australia and New Zealand National Water Quality Management Strategy (ANZECC and ARMCANZ 2000), however few toxicity test protocols with marine species are currently available in Australia. Limited toxicity test protocols with native marine species, such as macroalgal germination and cell division (Kevekordes and Clayton 1996), scallop larval development (Krassoi et al. 1996), sea urchin fertilisation (Simon and Laginestra 1997) and microalgal growth inhibition (Stauber et al. 1994) have been developed and applied to testing chemicals, effluents, leachates, surface waters and groundwaters in temperate regions. Tests with tropical and sub-tropical marine species such as corals, macroalgae and prawns are available; however, more protocols with rigorous quality assurance are required in order to sufficiently cover all taxonomic groups and geographic regions (ANZECC and ARMCANZ 2000).

Copepods are a diverse and abundant group of crustacean zooplankton, and are important in the diet of larger invertebrates, fish and mammals (Rippingale and Payne 2001; Schipp et al. 1999). They are widely distributed in marine and freshwater habitats, from polar to tropical environments, with an immense vertical range from 10 000 m below the sea surface to altitudes of 55 000 m in the Himalayas (Huys and Boxshall 1991). Copepods fulfill many of the desirable criteria for use as toxicity test species (Cairns 1982), and are particularly sensitive to contaminants. Several species such as Acartia tonsa, Acartia clause and Tisbe holothuariae have been reported to be sensitive to copper, with LC50 values for adults ranging from 10 to 105 µg Cu/L (Sosnowski and Gentil 1978; Moraitou-Apostolopoulou and Verriopoulos 1982). Despite this, only one species of copepod, Glaucodioufers imparipes, has been used in temperate Western Australia for toxicity testing (Woodworth, pers. comm.).

Acartia sinjiensis is a calanoid copepod that is widely distributed in sub-tropical and tropical waters of the Western Pacific from Japan (Ueda and Hiromi 1987) to Brisbane (Bayley 1965). It feeds on phytoplankton and detritus in the water column and completes its life cycle from naupliar to adult within 10 to 12 days (Willis 1999), making it potentially suitable for the development of both acute and chronic (whole life cycle) tests. It is also amenable to culturing in the laboratory, provided that food sources are tailored to each particular life stage. The microalga Isochrysis sp. is a suitable food for early life stages due to its small size and high unsaturated fatty acid content (Rippingale and Payne 2001), while larger cryptomonads such as Rhodomonas sp. are suitable for feeding adults (Schipp et al. 1999).

The aim of this study was to develop an acute toxicity test protocol with the native sub-tropical copepod Acartia sinjiensis suitable for determining the toxicity of chemicals and complex effluents. Optimisation of culture and test conditions was undertaken, and the reproducibility and sensitivity of the test was compared with other copepods and other marine species.

MATERIALS AND METHODS

General laboratory techniques and reagents

All laboratory glassware was pre-soaked in 10% nitric acid overnight and thoroughly rinsed with Milli-Q water (Millipore Corp, USA) prior to use. All chemicals used in culture preparation and toxicity testing were AR grade. Clean seawater used for culturing algae and copepods and as toxicity test diluent was collected in high density polyethylene containers offshore from Cronulla, NSW, and immediately filtered through a 0.45 µm cartridge filter which had been pre-rinsed with 1 L of 10% nitric acid and 10 L of Milli-Q water. Filtered seawater was stored in the dark at 4°C for a maximum period of one month.
Test organisms

The copepod Acartia sinjiensis was originally isolated from plankton collections offshore from Townsville, Queensland and for this study was obtained from mass cultures maintained at the Queensland Department of Primary Industries Northern Fisheries Centre (NFC), Cairns, Queensland.

On receipt at CSIRO, Sydney, the cultures were maintained in filled 1-L glass beakers, with slow aeration (1 bubble/sec) at 27°C and a salinity range of 30 to 35‰. Copepods were fed daily with two microalgae; an unidentifiable cryptomonad (CS-412) at 20000 cells/mL and Isochrysis aff. galbana (CS-177/3) (25 000 cells/mL), both obtained from the CSIRO Microalgal Culture Collection, Hobart. The cryptomonad and Isochrysis galbana were cultured in full-strength and half-strength f medium respectively (Guillard 1975) on a 12 h light:12 h dark cycle (cool white fluorescent tubes at 10-25 µmol/m²/s) at 25°C.

New copepod cultures were established by siphoning juvenile copepods (nauplii and copepodids) out of existing cultures using plastic tubing covered with 200 µm mesh and then were allowed to develop into adults. Cultures were maintained by replacing half the water volume (500 mL) three times per week. Debris was collected and discarded using a 5 mL plastic pipette.

To supplement cultures for ecotoxicological testing, copepods were also obtained from NFC each week. When received, juvenile copepods were removed and the remaining adults were slowly acclimated to the testing conditions (Table 1) over a four day period before being used in the toxicity tests. Only adult copepods (>10 days old) were used in all tests and copepods were fed 2 h prior to test set up to minimise the period without food.

Toxicity test procedure

A summary of the acute toxicity test protocol developed, modified from overseas test protocols for other marine copepods (ISO 1999; Kurk and Wollenberger 1999), is given in Table 1. The test endpoint was immobilisation after 24 and 48 h.

Eighteen millilitres of each of five concentrations of toxicant, together with seawater controls, were prepared in clean 20 mL glass scintillation vials, pre-silanised with Coatasil (BDH, Australia) to reduce adsorption of toxicants to the glass surfaces. For the toxicity tests, nine replicates were set up for each treatment: three replicates were used for the measurement of physicochemical parameters (pH, dissolved oxygen, salinity and temperature) at 0, 24 and 48 h (no copepods added); two replicates were combined for chemical analysis of the toxicant at 0 and 48 h (no copepods added); and four replicates were used to assess copepod immobilisation after 24 and 48 h.

Five adult copepods were carefully transferred to each labeled test vial using a glass Pasteur pipette, giving a total of 20 copepods per treatment. Each vial was individually covered with laboratory Parafilm and randomly placed in an incubation cabinet maintained at 27 ± 1°C on a 12 h light:12 h dark cycle at < 8 µmol/m²/s cool white fluorescent light. At 24 and 48 h intervals, the vials were randomly examined under a Maggie magnifying lamp for copepod immobilisation, which was determined by visual observations. Tests were considered acceptable if there was >80% mobilisation and <20% variability in controls.
Determining test sensitivity to copper, ammonia and phenol

The sensitivity of *A. sinjiensis* to three reference toxicants, copper (1 to 100 µg/L), ammonia (0.1 to 70 mg/L) and phenol (0.4 to 100 mg/L), was determined in at least four toxicity tests for each chemical.

A copper stock solution (100 mg Cu/L, from copper sulfate) was prepared in Milli-Q water and acidified using 1% HCl. The concentration of copper in the stock solution and in each test treatment was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Spectroflame EOP, SPECTRO Analytical Instruments) calibrated against standards prepared in clean seawater. All samples were acidified to 2% (v/v) with high purity nitric acid. Spike-recovery tests and analysis blanks were undertaken as part of the quality control procedures. The ICP-AES method had a detection limit of 3 µg/L of copper.

The ammonia stock solution was prepared by dissolving 6.11 g of ammonium chloride (May and Baker Ltd) in 100 mL Milli-Q water volumetrically to give a final concentration of 16 g/L. Ammonia in the stock solution and in each test treatment was determined using a Merck Spectroquant Kit (14752).

A 20 g/L phenol stock solution was prepared by dissolving 2 g of phenol (BDH) in 100 mL of Milli-Q water. Phenol was determined using a modified spectrophotometric method (Holme and Peck 1998). Samples were prepared by the addition of 1 mL of sample to 9 mL of 5% sodium chloride and 1 mL of Folin's reagent. The mixtures were left for 15 min for colour development and the absorbance determined at 620 nm on a UV-Visible Spectrophotometer (Shimadzu, Mini 1240).

**Application of the toxicity test to assessing the toxicity of complex effluents**

The toxicity test procedure developed was used to assess the acute toxicity of a leachate. A leachate pond sample was obtained from Bicentennial Park, Homebush Bay, Sydney, NSW, through the Sydney Olympic Park Authority, who operated a brickyard and tip in the area (Binet et al. 2003). Leachate from this site had previously been shown to contain high ammonia concentrations (114 to 137 mg total ammonia N/L) (Binet et al. 2003). The concentration of copper in the stock solution and in each test treatment was determined using a Merck Spectroquant Kit (14752).

Leachate samples were collected using a 2 m pole which held a pre-cleaned 1 L HDPE bottle. A total of 8 L of sample was collected. Samples were transported on ice to the laboratory and stored at 4°C, with toxicity testing commencing within 48 h of sample collection. The ammonia concentration in the leachate was measured immediately, and the leachate was diluted 1:4 with seawater. The salinity of the leachate was adjusted to 35‰ by the addition of artificial sea salts (USEPA 1994). The sample was serially diluted (1:2) in seawater to give concentrations ranging from 0 to 25%. Controls included a seawater control and an artificial sea salt control. The test procedure was the same as that outlined previously.

**Statistical analyses**

The EC50 value, i.e. the toxicant concentration that caused immobilisation in 50% of the exposed copepods was estimated in each toxicity test using the Trimmed Spearman-Karber method in ToxCalc Version 5.0.23. The Lowest Observable Effect Concentration (LOEC) and the No Observable Effect Concentration (NOEC) were estimated in each test using the Wilcoxon Rank Sum or Dunnett's Tests, after checking for data normality and homogeneous variance. Curve fits were done using Origin Version 3.5 software.

**Comparison of sensitivity of *Acartia sinjiensis* to other species using species sensitivity distributions**

The sensitivity of *A. sinjiensis* to copper, ammonia and phenol was compared to that of other species e.g. algae, invertebrates (including copepods) and fish using quality checked data from the ANZECC and ARMCANZ (2000) water quality guidelines database. Acute data (LC/EC50 values for mortality or immobilisation for 48 h exposures only to each toxicant) were combined with EC50 data generated from this study in species sensitivity distributions, according to the ANZECC and ARMCANZ (2000) approach. Log-normal, log-logistic and Burr Type III curve distributions were fitted to plots of cumulative probability versus toxicant concentration.

**RESULTS AND DISCUSSION**

During method development immobilisation of copepods under controlled conditions ranged from 0 to 70%. This variability was overcome by lowering the salinity (between 27 and 30‰) and light intensity (<1 µmol/m²/s) for copepod culture maintenance, reducing copepod holding times without food prior to test commencement, careful handling during copepod transfer to test solutions and the use of new test vials for each treatment. The test acceptability criterion for controls was set at 80% mobilisation to account for this variability.

**Test sensitivity**

The sensitivity of *A. sinjiensis* to the three reference toxicants—copper, ammonia and phenol, was determined for measured concentrations of these toxicants over 24 and 48 h and is summarised in Table 2.

**Copper**

*A. sinjiensis* was sensitive to copper, with 24- and 48-h EC50 values ranging from 24 to 50 µg Cu/L and 15 to 24 µg Cu/L, respectively. Combined copper concentration response curves for both 24 and 48 h gave a 24 h EC50 (95% CL) of 43 (34 to 54) µg Cu/L and a 48 h EC50 of 21 (16 to 26) µg Cu/L (Figure 1). The LOEC was 13 µg Cu/L for both exposure periods.

Reported 48-h EC50 values for adult copepods range from 10 to 105 µg Cu/L (Moraitou-Apostolopoulou 1978; Madhupratap et al. 1981; Moraitou-Apostolopoulou and Verriopoulos 1982, Hall et al. 1997). Only one species (*Acartia spincicauda*) was more sensitive to copper (48 h EC50 of 10 µg Cu/L) (Madhupratap et al. 1981) than *A. sinjiensis* (48 h EC50 of 21 µg Cu/L) in the present study.
Table 2. EC50, LOEC and NOEC values after 24 and 48 h exposure of *Acartia sinjiensis* to ammonia, copper and phenol.

<table>
<thead>
<tr>
<th>Toxicant (mg/L)</th>
<th>24 h EC50 (95%CL)</th>
<th>LOEC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NOEC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>48 h EC50 (95%CL)</th>
<th>LOEC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NOEC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>31 (25-38)</td>
<td>5</td>
<td>1</td>
<td>10 (8-12)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Copper</td>
<td>0.043 (0.034-0.054)</td>
<td>0.013</td>
<td>&lt;0.013</td>
<td>0.021 (0.016-0.026)</td>
<td>0.013</td>
<td>&lt;0.013</td>
</tr>
<tr>
<td>Phenol</td>
<td>49 (39-61)</td>
<td>25</td>
<td>6.3</td>
<td>20 (16-25)</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Geometric mean of 4 tests

<sup>b</sup> LOEC > EC50 therefore not reported

Figure 1. Concentration response curves expressed as immobilisation (%) versus copper concentrations (μg/L) at (a) 24 h and (b) 48 h.
Using all the ANZECC and ARMCANZ (2000) acute data for 48 h copper exposure, it appears that *A. sinjiensis* is the second most sensitive species to copper. Indeed crustaceans, and copepods in particular, comprised the 12 most sensitive species to copper in the species sensitivity distribution (Figure 2).

**Ammonia**
*A. sinjiensis* was much less sensitive to ammonia than to copper, with 24 and 48 h EC50 values ranging from 11 to 92 mg/L and 6 to 15 mg/L respectively. EC50 values derived from the combined ammonia concentration response curves (Figure 3) were 31 (25 to 38) mg N/L and 10 (8 to 12) mg N/L for a 24 - and 48 h exposure respectively, with a LOEC of 5 mg N/L and NOEC of 1 mg N/L for both exposure times. Of the total ammonia added, about 5% was present in the more toxic unionized form at the test temperature of 27°C and pH of 7.8.

Comparisons of ammonia sensitivity between species are difficult due to the different test conditions (pH and temperature) used. Ammonia toxicity increases with increasing temperature and pH, due to the increase in unionized ammonia. Very few studies have examined the acute toxicity of ammonia to adult copepods. Linden et al. (1979) reported a 96 h EC50 of 70 mg/L ammonia for the copepod *Nitocra spinipes*, suggesting it is considerably less sensitive than *Acartia sinjiensis*. EC50 values for other crustaceans such as the redtail prawn (*Penaeus penicillatus*), tiger prawns (*P. monodon*) and the fl eshy prawn (*P. chinensis*), ranged from 1.2 to 3.1 mg ammonia (Chen and Lin 1991, 1992; Chen1990a,b). These values are substantially lower then the EC50 obtained for *Acartia sinjiensis* in this study. *Acartia sinjiensis* was also one of the least sensitive species to ammonia compared to other invertebrates and fish (Figure 4), despite the fact that a large proportion of the ammonia would have been present in the toxic unionized form.

**Phenol**
Of the three chemicals tested, phenol was the least toxic to *A. sinjiensis*, with 24 and 48 h EC50 values ranging from 33 to 62 mg/L and 12 to 41 mg/L respectively. EC50 values derived from the combined phenol concentration response curves (Figure 5) were 49 (39 to 61) mg/L and 20 (16 to 25) mg/L for 24 and 48 h exposure respectively, with a NOEC of 6 mg/L for both exposure times. *A. sinjiensis* was more sensitive to phenol than the adult *Acartia clause*, with 24 h EC50 values for phenol of 49 mg/L and 323 mg/L respectively (Buttino 1994).

A comparison of the sensitivity of *A. sinjiensis* with other biota (Figure 6) shows that this species was more sensitive than other crustaceans to phenol, except for the grass shrimp (EC50 of 4.7 mg/L, Tatem et al.1978). *A. sinjiensis* fell midway between the most sensitive marine species (tigerfish, 48 h EC50 of 4.4 mg/L, Dange and Masurekar, 1984) and the least sensitive species (Aesop shrimp 48 h EC50 of 175 mg/L, Portmann and Willis 1971).

**Test reproducibility**
Within-test precision was good for each toxicant, with coefficients of variation in the controls of <20%. Between test precision was 25% for copper, which was within the recommended limit of <30% (Environment Canada 1990). However, coefficients of variation between tests for ammonia (35%) and phenol (50%) exceeded the recommended limit. Further assessment of the test’s reproducibility for these toxicants is required.

**Toxicity of leachate**
The leachate sample collected from Bicentennial Park had a measured ammonia concentration of 120 mg N/L. Based on the above sensitivity of *A. sinjiensis* to ammonia alone, the predicted toxicity of the leachate (due to ammonia) was 120/31 = 3.9 Toxic Units (TU) and 120/10 = 12 TU for 24 and 48 h respectively. The determined 24 - and 48 h
Figure 3. Concentration response curves expressed as immobilsation (%) versus ammonia concentration (mg/L) at (a) 24 h and (b) 48 h.

Figure 4. Ammonia species sensitivity distribution (48 h acute data only taken from ANZECC/ARMCANZ(2000)).
Figure 5. Concentration response curves expressed as immobilisation (%) versus phenol concentrations (mg/L) at (a) 24 h and (b) 48 h.

Figure 6. Phenol species sensitivity distribution (48 h acute data only taken from ANZEC/ ARMCANZ(2000)).
EC50 values were 18% (CL-16 to 21) and 5% (C.L-4 to 7) leachate respectively, giving an observed toxicity of 100/18 = 5.6 (± 3) TU and 100/5 = 20 (±2) TU respectively. While the observed and predicted toxicities were similar after 24 h (3.9 PTU versus 5.6 ± 3 OTU), indicating that ammonia was possibly a major toxicant, the poor concurrence of the observed and predicted toxicities at 48 h (12 PTU versus 20 ± 2 OTU) may suggest that some additional toxicant may be contributing to toxicity. Binet et al. (2003) also found that ammonia was the major toxicant in saline leachate samples collected from several sites at Homebush Bay, including Bicentennial Park.

CONCLUSIONS
An acute toxicity test based on immobilisation in the tropical/subtropical marine copepod A. sinjiensis was successfully developed. The acute A. sinjiensis test was particularly sensitive to copper in comparison to other copepod species and other marine biota, of intermediate sensitivity to phenol, and was relatively insensitive to ammonia.

A. sinjiensis is an ideal tropical test organism for the further development of a chronic test because it can be easily cultured in the laboratory and has a short life cycle.

ACKNOWLEDGMENTS
The authors would like to thank Gale Semmens (Northern Fisheries Centre, Cairns) for provision of the copepod culture; Edwina Laginiestra from the Sydney Olympic Park Authority for funding and provision of the leachate sample; and Merrin Adams, Monique Binet, Janine Wech (CSIRO Land and Water), Nicola Creighton (ANSTO Environmental Division) and Peta Hunt (Sinclair Knight Merz Ecotoxicology Laboratory) for technical assistance and advice throughout the project. We would also like to thank Bill Booth (Department of Cell and Molecular Biology, University of Technology Sydney) for help with phenol analyses.

REFERENCES


Chen JC and Lin CY. 1991. Lethal effects of ammonia and nitrate on Penaeus penicillatus juveniles at two salinity levels. Comparative Biochemistry and Physiology 100, 477-482.


Dange AD and Masurekar VB. 1984. Acute toxicity of petroleum hydrocarbons to the estuarine fish Therapon jarbua (Forsskal) and the estuarine clam Katelysia opima (Gmelin). In Proceedings, Symposium on Coastal Aquaculture, 828-832.


Linden E, Bengtsson BE, Svanberg O and Sundstorm G. 1979. The acute toxicity of 78 chemicals and pesticide formulations against two brackish water organisms, the Bleak (Alburnus alburnus) and the Harpacticoid. Chemosphere 8, 843-851.


Portmann JE and Willis KW. 1971. The Toxicity of 140 Substances to the Brown Shrimp and Other Marine Animals Shellfish. Information Leaflet No. 22 (2nd ed.). Ministry of Agriculture and Fish Food Lab, Burnham-on-Crouch, Essex, and Fish Exp. Station Conway, North Wales 1-12.


