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An assessment of three Harpacticoid Copepod species for use in ecotoxicological testing

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Abstract

The relatively short life cycles of harpacticoid copepods makes them appropriate animals for use in tests that rapidly assess the #180, sublethal, or chronic effects of sediment contaminants. In this study, four harpacticoid copepod species (*Nitocra spinipes, Tisbe tenuimana, Robertgurneya hopkinsi, and Halectinosoma* sp.) were isolated from clean marine sediments, and procedures for laboratory culturing were developed. *Halectinosoma* sp. was abandoned due to handling difficulties. For the remaining species, the influence of food type and quantity on life-cycle progression was assessed. A mixed diet, comprising two species of algae (*Tetraselmis* sp. and *Isochrysis* sp.) and fish food (Sera Micron) was found to maintain healthy cultures and was fed during laboratory tests. Water-only exposure to dissolved copper (Cu) showed that the times (range) required to cause 50% lethality (LT_{50}) were 24 (22-27) h at 50 mu g Cu/l for *T. tenuimana*; 114 (100-131) and 36 (32-40) h for 200 and 400 mu g Cu/l, respectively, for *N. spinipes*; and 119 (71-201) and 25 (18-33) h for 200 and 400 mu g Cu/l, respectively, for *R. hopkinsi*. 96-h LC(50) (concentration causing 50% lethality) were also determined for adult *N. spinipes* exposed to cadmium, copper, zinc, ammonia, and phenol. A ranking system was generated based on the ease handling and culturing, rate of maturity, food selectivity and sensitivity to Cu. From this ranking, *N. spinipes* was determined to be the most suitable species for use in developing sediment-toxicity tests. The measurement of total reproductive output of *N. spinipes* during 10-day exposure to whole sediment was found to provide a useful end point for assessing the effects of sediment contamination."

Keywords

assessment, three, Harpacticoid, Copepod, species, for, use, ecotoxicological, testing, CMMB

Disciplines

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An assessment of three harpacticoid copepod species for use in ecotoxicological testing

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Keywords: harpacticoid copepod, chronic, sediment toxicity, ecotoxicology, culturing, *Nitocra spinipes*

Abstract

The relatively short life-cycles of harpacticoid copepods makes them appropriate animals for use in tests that rapidly assess the acute, sublethal or chronic effects of sediment contaminants. In this study, four harpacticoid copepod species (*Nitocra spinipes*, *Tisbe tenuimana, Robertgurneya hopkinsi* and *Halectinosoma* sp.) were isolated from clean marine sediments, and procedures for laboratory culturing were developed. *Halectinosoma* sp. was abandoned due to handling difficulties. For the remaining species, the influence of food type and quantity on lifecycle progression was assessed. A mixed diet, comprising two algae (*Tetraselmis* sp. and *Isochrysis* sp.) and fish food (Sera® Micron) was found to maintain healthy cultures and was fed during laboratory tests. Water-only exposure to dissolved copper showed that the times required to cause 50% lethality (LT_{50}) were: 24 (22-27) h at 50 µg Cu/L for *T. tenuimana*; 114 (100-131) h and 36 (32-40) h for 200, and 400 µg Cu/L, respectively, for *N. spinipes*, and 119 (71-201) h and 25 (18-33) h for 200 and 400 µg Cu/L, respectively, for *R. hopkinsi*. 96-h lethal effects thresholds were also determined for adult *N. spinipes* exposed to Cd, Cu, Zn, ammonia and phenol. A ranking system was generated based on the ease handling and culturing, rate of maturity, food selectivity and sensitivity to copper. From this ranking, *N. spinipes* was determined to be the most suitable species for use in developing sediment toxicity tests. The measurement of total reproductive output of *N. spinipes* over 10-days exposure to whole sediment was found to provide a useful endpoint for assessing the effects of sediment contamination.

Introduction

Sediments act as a sink for many aquatic contaminants (Linnik and Zubenko 2000). To assess and manage contaminated sediments in coastal marine environments (such as estuaries), toxicity testing is performed. These tests identify the potential effects of the sediment-associated contaminants on benthic organisms (Simpson et al. 2005). While a wide range of laboratory-based sediment toxicity tests are available that assess acute effects on benthic organisms (Hagopian-Schlekat et al. 2001; Adams and Stauber 2004; Schipper et al. 2008), far fewer tests are available to assess sub-lethal or chronic effects (Scarlett et al. 2007; van den Heuvel-Greve et al. 2007; Mann et al. 2009).

Chronic toxicity tests utilise exposure periods which last for a longer component of an organisms life-cycle than acute tests. However, while acute toxicity tests generally have a time advantage over chronic tests, short exposure periods may not always provide an adequate assessment of effects (Finkelstein and Kern 2005; Scarlett et al. 2007). Typical chronic test endpoints include embryo development, growth, moulting and reproduction (Scarlett et al. 2007; Greenstein et al. 2008). Advantages of chronic tests may include greater ecological relevance, protection at the population level, increased sensitivity, better prediction of toxicity, and the ability to use results for modelling contaminant effects on populations dynamics (Finkelstein and Kern 2005; Smit et al. 2006; van den Heuvel-Greve et al. 2007; Kennedy et al. 2009). For example, increased sensitivity following chronic exposures (compared to acute lethality tests) has been observed for amphipods (Castro et al. 2006; Scarlett et al. 2007), copepods (Bejarano et al. 2004) and polychaete worms (Rice et al. 1995; Moreira et al. 2005). However, chronic tests are not always more sensitive than acute methods (McGee et al. 2004; Greenstein et al. 2008), and long test durations often result in greater variability in the performance of these tests (McGee et al. 2004; Greenstein et al. 2008; Kennedy et al. 2009). The resulting increased variability will usually require greater test replication to meet quality control criteria, and this increases labour intensiveness and costs (Kennedy et al. 2009).

Ideally, sediment bioassays should be rapid, easy to conduct and inexpensive. Criteria for selecting an appropriate test species should also include sensitivity to contaminants, year-round availability from natural populations or laboratory cultures, ecological significance of the species and species distribution (ASTM 2003). Despite the many advantages of chronic tests, the disadvantage caused by the long test duration has resulted in acute toxicity methods remaining as the favoured tests for most sediment quality assessments (ASTM 2003; Simpson

et al. 2005). It is therefore desirable that rapid methods for assessing chronic effects be developed.

As harpacticoid copepods are abundant in most sediment environments, are closely associated with sediments, and have relatively short life-cycles, they appear to be excellent candidate species for use in tests that rapidly assess acute, sublethal or chronic effects of sediment contaminants (Bengtsson 1978; Brown et al. 2005). For water-only testing, standard testing methods are available using the harpacticoid species, *Amphiascus tenuiremis* (ASTM 2004). A significant technical issue for using harpacticoid copepods in routine sediment toxicity tests is their small size, typically adults body lengths are <0.6 mm, which often makes it difficult to thoroughly and rapidly isolate the organisms from sediments. While *A. tenuiremis* is also used in sediment toxicity tests (Chandler and Green 1996; Chandler and Green 2001; Hagopian-Schlekat *et al.* 2001), the development of ecotoxicology tests that use a wider range of harpacticoid species will improve how we assess and manage contaminated sediments.

This study investigated the suitability of the harpacticoid copepod species *Nitocra spinipes*, *Tisbe tenuimana*, *Robertgurneya hopkinsi* and *Halectinosoma* sp. (isolated from sediments in south-eastern Australia) for use in ecotoxicology. Laboratory culturing procedures were developed and the influence of food type on lifecycle progression (development) assessed. Time-to-death experiments were used to assess the sensitivity of each species to copper in water-only bioassays. The copepods ease of handling, culturing, rate of maturity, food selectivity and sensitivity to dissolved copper was used to rank the suitability of each species for developing toxicity test methods. Based on these experiments, the adult *N. spinipes* 96-h lethal effects thresholds were determined for Cd, Cu, Zn, ammonia and phenol in water-only exposures. Whole-sediment exposures were then used to assess the sensitivity of *N. spinipes* reproduction to sediments collected from the field with a range of contaminant concentrations and properties.

Methods

Water and sediments

Seawater with salinity ranging from 30 to 34 PSU was collected from Port Hacking, Sydney, Australia, membrane filtered (0.45 µm, MiniSart, Sartorius, Oakleigh, VIC, Australia), and acclimated to the room temperature of 21±1°C. Where necessary, the salinity of the filtered seawater was adjusted to the test salinity of 30 PSU using Milli-Q deionised

98 water (18 MΩ/cm; Milli-Q Academic Water System, Sydney, Australia). Long-term use of
99 this seawater source had indicated that it did not contain any contaminants at concentrations
100 of concern for ecotoxicology tests.

101 Control sediments were collected from a range of estuarine sites that had been
102 characterised and shown to have low or negligible concentrations of metal and organic
103 contaminants (Simpson et al. 2004). The surface layers (upper 2-4 cm) of sediments were
104 collected using clean Teflon spatulas and press-sieved through a 1.1-mm mesh on site to
105 remove coarse materials. The sediment was transferred into clean plastic bags with minimal
106 headspace and stored in a cool room at 4 ºC for no longer than one month. Clean sand that
107 contained negligible contamination (Spadaro et al., 2008) was also used as a control material.

108 Contaminated sediments were collected from estuarine sites of unspecified locations,
109 stored at 4ºC in the dark and toxicity testing undertaken within 8 weeks. Analyses of physico-
110 chemical properties (pH, organic carbon, particle size, acid-volatile sulfide) and metal
111 contaminants were made on all sediments collected. Concentrations of total petroleum
112 hydrocarbons were <250 mg/kg and polycyclic aromatic hydrocarbons were <1 mg/kg in all
113 sediments.

114 *Copepod collection and culturing*

115 Copepods were isolated from field collected sediments sampled from Grays Point and
116 Bonnet Bay, Sydney, NSW Australia. Sediments were sieved using a 250 µm mesh to
117 remove large particles and debris. Adult copepods were isolated from the <250 µm sediment
118 under a light microscope using a plastic Pasteur pipette.

119 At least four morphologically distinct copepods with high abundance were easily
120 identified from the mixtures of meiofauna isolated from field sediments, and were considered
121 to represent four species. Initial cultures of these species were started using 100-200
122 individuals, and copepods whose morphology could not be matched to these species groups
123 were removed and discarded. Copepod cultures were established in clean plastic containers
124 (20 × 14 × 10 cm) with a 0.5 – 1 cm layer of a mixture of silty sediment and clean sand
125 (~50% particle size < 63 µm) with weekly water changes (filtered seawater). All four species
126 cultured successfully, and after one month the adult copepods were sieved (50 µm nylon
127 sieve) from each culture and used to commence new cultures. Juveniles, nauplii, copepodites
128 and any copepods that did not match the morphology of the selected species were discarded

during this process in order to purify the cultures. This was repeated again after two months and then specimens of each cultured copepod species taken for taxonomic identification.

Nematodes became present in high numbers in many of the cultures and required monitoring and removal as they were likely to compete with copepods for food resources. The nematodes or their larvae were presumably either present in the sediments or seawater. New cultures were therefore initiated using sediment and seawater that had been sterilized by autoclaving 30 minutes at 120 ºC (AA16, Laboratory Equipment Pty Ltd, Sydney, Australia) to eliminate pre-existing meiofauna.

Filtered seawater at 30 PSU was added to the cultures to a depth of approximately 10 cm. The water was continuously aerated and renewed weekly. Cultures and bioassays were maintained in a temperature controlled laboratory (21 ± 2 °C) under ambient light conditions. The copepod cultures were initially fed approximately 30 ml of a 1:1:1 mix of three algae species (*Tetraselmis sp.* (CS-87)*, Phaeodactylum tricornutum* Bohlin (CS-29/4) and *Isochrysis* sp. (CS-177), total concentration 1×10^7 cells/ml) twice weekly. New copepod cultures were initiated every 2-3 weeks by transferring 100 gravid females from the existing cultures to new culture containers.

Adult copepods were removed from cultures using a plastic Pasteur pipette to gently suck up aliquots from the surface sediment, which were passed through a 180 µm sieve, retaining adults while letting most of the sediment, debris and juveniles pass through the mesh back into the culture container. Adults were transferred from the sieve (using clean seawater) into a Petri dish, placed under a light microscope (maximum 36× magnification) and individual copepods were sorted based on maturity, gender and the presence of egg sacs using a pasteur pipette.

Influence of food type and quantity on copepod culturing

To determine the influence of food type and quantity on the reproduction and development of copepods over 7 days, feeding experiments were conducted using two diets at three food concentrations. The first was a 1:1:1 mix of three planktonic algal species, *Phaeodactylum tricornutum., Tetraselmis* sp.*, Isochrysis* sp. (total cell count of 1×10^7 cells/ml) added to the vials at 0, 1, 3 and 10×10^6 cells per feed. The second diet was a powered fish food (Sera® Micron) added to vials at 0, 0.15, 0.50 and 1.5 mg per feed. This fish food comprised a variety of dried marine algae, plants, fish and other protein sources to give a composition of approximately 50.2%, 8.1%, and 9.2% of protein, fat and fibre,

161 respectively. During experiments, five gravid female copepods were placed in 5 ml
162 polycarbonate vials containing clean filtered seawater. Food was added to each vial at time
163 zero then again after each water change. The overlying water was exchanged every second
164 day using a plastic Pasteur pipette to remove the overlying water in 1-2 mL portions. This
165 water was checked under the microscope and any living nauplii, copepodite or adult copepods
166 were returned to the test vial. This ensured that the loss of biota during the test period due to
167 handling was kept to a minimum. At the end of the 7-day test the number of nauplii,
168 copepodite and adult copepods was recorded.

169 *Toxicity test procedures*

170 The sensitivity of the cultured copepods to dissolved copper was determined using
171 time-to-death bioassays for adult copepods. Organisms were exposed to concentrations of 0
172 to 800 µg Cu/L in 0.45 µm filtered seawater prepared from $CuSO_4 \cdot 5H_2O$ (BDH Laboratory
173 Supplies). Fifteen adult copepods (excluding gravid females) were transferred to clean plastic
174 Petri dishes (diameter of 50 mm) containing 10 mL of the exposure solution. The copepods
175 were not fed during these bioassays. Tests were observed twice daily by light microscope and
176 the number of living copepods was recorded. The experiment was terminated when no
177 surviving copepods could be found in the treatment, typically 4 to 7 d. LT_{50} values (time (h)
178 to cause 50% mortality) and 95% confidence limits (95% CL) were calculated based on
179 nominal copper concentrations.

180 To determine the effects of ammonia, cadmium, copper, zinc, and phenol on adult *N.*
181 *spinipes* survival, nominal concentrations were achieved by adding the appropriate volume of
182 seawater stock solutions of ammonia (1.4 g total NH_4NO_3/L), 100 g/L metal sulfate, or 10 g/L
183 phenol (AR grade, BDH Laboratory Supplies) to test beakers. The 4-day survival of adults
184 was determined using three replicates of 10 organisms at six nominal concentrations of each
185 chemical. After 4 days of exposure the survivors were enumerated for each test vessel.
186 Filtered (<0.45 µm) water samples were taken at the start and completion of the tests and
187 ammonia or dissolved metals were determined. The calculations of LC50s (concentration that
188 causes 50% lethality) were based on measured concentrations of copper and ammonia, but
189 nominal concentrations were used for phenol.

190 *Whole-sediment exposures using N. spinipes*

191 The influence of sediment properties and contaminants on the reproductive output of
192 *N. spinipes* was assessed following exposure to undiluted test sediments over a 10-day period.

193 Approximately 0.5 g of test sediment and ~9 mL of filtered seawater (30 PSU) was added to a
194 10 ml polycarbonate vial (10-mm diameter, 10-cm height), then each vial was incubated at
195 21ºC overnight to allow sediments to settle. The following day, overlying water was replaced
196 and five gravid females (3-5 weeks old) were randomly assigned to each vial. For most tests,
197 there were five replicates of each sediment. For several tests, six replicates were used for
198 each sediments tested. All tests were undertaken at 21±1ºC with a 12 h light, 12 h dark cycle.
199 Overlying water concentrations of metals and ammonia, along with physico-chemical
200 parameters (temperature, pH, salinity and dissolved oxygen) were measured twice during the
201 10-day test. At the completion of the test the number of nauplii (first juvenile life stage of the
202 copepod) and copepodites (second juvenile life stage) in each vial was recorded by
203 microscopy. The presence of sediment particulates made counting more difficult, however
204 suitable recoveries were achieved by gently agitating the surface of the sediment and pipetting
205 it into a petri dish using filtered seawater and counting these organisms. The remaining
206 sediment from each treatment was then passed through a 20 µm sieve and the retained
207 organisms transferred into another petri dish and counted. The organism numbers from both
208 of these counting steps were combined. The surface sediments contained 80-90% of the
209 organisms. Results were expressed as a percentage of the reproductive output (nauplii and
210 copepodites) in the control sediment. Treatments were fed a diet of 2×10^4 cells/ml of 1:1
211 *Isochrysis sp*. and *Tetraselmis sp*. (discussed below) as well as 0.3 mg Sera micron® fish food
212 (<63 µm) per test vial twice a week.

213 *Statistical Analysis*

214 LT_{50} values (time (h) to cause 50% mortality) and confidence limits were calculated
215 from logistic time–response curves for each copper concentration using a Microsoft Excel
216 (Redmond, WA, USA) spreadsheet (Barnes et al. 2003). Toxcalc for Microsoft Excel
217 (TidePool Scientific Software, Mckinleyville, CA) was used to perform the LC_{50}/EC_{50}
218 calculations, the probit function was used when the data could be adjusted to a normal
219 distribution. The remaining of LC_{50} (concentration causing 50% lethality) calculations were
220 obtained by using the Trimmed Spearman-Karber method (Finney 1978). t-tests were used to
221 determine if the response of the copepods in the test sediment was different to that in the
222 control sediment. The appropriate Student's t-test was preformed on the data to determine
223 significant differences between means of the different feeding concentrations and diet types.
224 Statistical analysis was performed in Microsoft Excel 2003 Data Analysis Tool Pack and

Solver add-in. Variances were determined to be either equal or unequal using the two sample F-test. Significance in all statistical tests was set at the $p<0.05$ level.

Analytical Methods

All chemicals were analytical reagent grade or equivalent analytical purity (BDH Laboratory Supplies, Poole, England, or Univar, Ajax Finechem, Sydney, Australia). Plasticware (made of polycarbonate or polyethylene) used for all tests and analyses was new or re-used following cleaning. Containers for analyses were cleaned by soaking in 10% (v/v) HNO_3 (Analytical Reagent grade) for a minimum of 24 h, followed by thorough rinsing with Milli-Q water. Glass beakers and acrylic beaker-lids used for toxicity tests were cleaned in a dishwasher (Gallay Scientific Pty Ltd, Melbourne, Australia) programmed for a phosphate-free detergent wash (Clean A, Gallay Scientific Pty Ltd) and a dilute acid wash (1% HNO_3), followed by thorough rinsing with Milli-Q water.

Measurements of pH were made using a pH probe and meter as described previously (Simpson et al. 2004). Salinity, temperature (YSI 30, Springs, OH, USA) and dissolved oxygen measurements (MI-730 Dip-type O_2 microelectrode and OM-4 oxygen meter, Microelectrodes Inc., Bedford, NH) were made in accordance with the instrument manufacturer's instructions. Samples for dissolved metals analyses were acidified with concentrated HNO_3 (2% volume/volume, Tracepur, Merck, Darmstadt, Germany) and concentrations determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES; Spectroflame EOP, Spectro Analytical Instruments, GmbH, Kleve, Germany) calibrated with matrix-matched standards (QCD Analysts). Method blanks, method duplicates and spike recoveries were performed on at least 10% of the filtered samples. Method blanks were below the 2-10 µg/L limits of reporting, duplicates within 15% and spike-recoveries were 85-110% for all metals. Methods for measurement of sediment particle size (by wet sieving through 63 µm nylon sieves followed by gravimetry), total organic carbon (OC, Dohrmann DC-190 TOC analyzer, Teledyne Tekmar, Mason, OH), acid-volatile sulfide (AVS) and porewater (PW) extraction (centrifugation at 800 g for 5 min) have been described previously (Simpson 2001; Spadaro et al. 2008). Dissolved ammonia was analysed colorimetrically using a Merck Spectroquant Kit (14752).

Results and Discussion

Copepod species and culturing

256 The four copepods in culture were identified as harpacticoid species *Nitocra spinipes*,
257 *Tisbe tenuimana, Robertgurneya hopkinsi* and *Halectinosoma* sp. The cultures of
258 *Halectinosoma* sp. were abandoned because of the small size of this species (304 ± 24 µm;
259 mean ± SD, n=5) and a low degree of movement which made subsequent handling of this
260 species difficult. The size of the harpacticoid copepods differed between species with body
261 lengths of adults (males and females) of 649 ± 72 µm for *N. spinipes*, 547 ± 15 µm for *T.*
262 *tenuimana* and 548 ± 19 µm for *R. hopkinsi*, respectively (average ± SD, n=5). (Photographs
263 provided in the Supporting information, Plate S1).

264 The copepods *N. spinipes*, *T. tenuimana* and *R. hopkinsi*, reproduced successfully in
265 sediment types ranging from silty (98% <63 µm) to sandy (29% <63 µm). However, it was
266 easier to isolate adult copepods from sediment when cultured in sediment that had been sieved
267 to <20 µm, as the sediment could be washed through a 20 µm sieve leaving behind the
268 copepods and juvenile copepod life stages (nauplii and copepodites). Water changes were
269 generally performed once per week, however the cultures remained viable even when longer
270 periods of time (up to 4 weeks) lapsed between water renewals.

271 *Affect of food type on reproduction and juvenile development*

272 Harpactiocoid copepods are known to feed on a range of food sources, including
273 bacteria, diatoms (algae) and detritus (De Troch *et al.* 2006). The quality and quantity of food
274 available to the copepods can have an impact on the growth, reproduction and mortality of
275 these organisms. The response of copepod reproduction and development to the presence of
276 food has previously been shown to depend on the nutritional value of the available food, the
277 ability of copepods to select the most nutritious source/s and their nutritional requirements
278 (Koski et al. 2006). This has been investigated previously for both pelagic (Tang and Taal
279 2005; Ismar et al. 2008; Saage et al. 2009) and harpacticoid species (Weiss et al. 1996;
280 Rhodes 2003; Dahl et al. 2009).

281 The use of diatoms as food by copepods is known to be influenced by cell size,
282 morphology and cellular composition as well as the morphology of the copepods mandibles
283 (Koski et al. 1998; De Troch et al. 2006). For calanoid copepods, it has been shown that
284 dietary diversity is important in promoting zooplankton production and ensuring a
285 nutritionally complete diet (Kleppel 1993; Anderson and Pond 2000). Similarly, Wyckmans
286 et al. (2007) demonstrated that offering a diverse diet to three species of harpacticoid copepod
287 resulted in the copepods feeding on a wider range of algae diatoms with a reduction in the
288 consumption of any one species. Wyckmans et al. (2007) also reported that the preference of

289 diatom type was species specific. This makes sense in terms of the 'optimal foraging' theory
290 which suggests that consumers will selectively feed on food resources that will maximise
291 energy intake (Hughes 1980). Also in accordance with the optimal foraging theory is the
292 observation that the grazing rate of harpacticoid copepods increases in response to an increase
293 in food availability both in the laboratory (De Troch et al. 2007) and in the field (Montagna et
294 al. 1995).

295 Based on these previous studies, we chose to trial a multi-species algal diet to culture
296 *N. spinipes*, *R. hopkinsi* and *T. tenuimana* in the laboratory. Three algal species fed to the
297 copepods were selected due to the small cell size and the availability of these species in our
298 laboratory. While no estimates were made of the grazing rate, or the species selectivity of the
299 copepods, it was found that the copepods had a positive response to the addition of food
300 which was measured as an increase in offspring production (Fig. 1). Furthermore, increasing
301 the biomass of the added food stock resulted in an increase in the reproductive output for the
302 three copepod species tested in this study.

303 Harpacticoid copepods are often detritus feeders (Norsker and Støttrup 1994) and are
304 therefore opportunistic feeders, suggesting that the use of artificial foods may also be suitable
305 to maintain laboratory cultures. Rhodes (2003) found that the use of a formulated food, which
306 was prepared from a mixture of vitamins, juice and brewer's yeast, was able to sustain
307 cultures of the harpacticoid copepod *Nitocra lacustris* without compromising the density or
308 growth rate of the cultures when compared to cultures fed live algae. Therefore we also chose
309 to trial a commercially available powdered fish food (Sera® Micron) as a source of nutrition
310 for the copepods (Fig. 1). For *N. spinipes* and *R. hopkinsi*, the use of a small amount of
311 powdered fish food was shown to result in an increase in offspring production, which was not
312 significantly different from the highest algal feed treatments.

313 Higher reproductive output was observed for all three copepod species when fed the
314 mixed algal diet in comparison to the powdered fish food (Fig. 1). The number of nauplii that
315 hatched during the 7-day period generally increased with the addition of higher concentrations
316 of algae, up to 10×10^6 cells/feed for *N. spinipes* (Fig. 1a), and to 3×10^6 cells/feed for *T.*
317 *tenuimana* (Fig. 1b) and *R. hopkinsi* (Fig. 1c). A similar trend was observed for the
318 development of juveniles (nauplii into copepodites) for the species *N. spinipes* and *T.*
319 *tenuimana* in which there were no copepodites present in the control treatments (no food) for
320 either species, however their numbers increased with the addition of food up to the high and
321 medium treatments, respectively (Fig. 1a and b). For *R. hopkinsi*, no copepodites were

observed throughout the experiments (Fig. 1c and f), possibly due to the longer life-cycle of this species (approximately 35 days nauplii (F_0)-nauplii (F_1) determined for *R. hopkinsi* compared to approximately 28 and 24 days determined for *T. tenuimana* and *N. spinipes*, respectively).

When fed powdered fish food, the reproductive output varied between the three species. For *N. spinipes,* the addition of low and medium concentrations of powdered fish food resulted in a significant increase in the number of nauplii hatching, however the number of copepodites was greater in the low concentration treatment ($p<0.01$) (Fig. 1d). The addition of the low concentration of fish food to *T. tenuimana* resulted in a decrease in the number of nauplii, but compared to the controls, there was a significant increase in the number of copepodites. For the high fish food treatment, the number of nauplii and copepodites significantly decreased (Fig. 1e). The number of nauplii produced by *R. hopkinsi* increased when provided the lowest concentration of fish food, however there was a significant decrease in reproductive output when a greater concentration of fish food was provided. As with the algae treatment, no copepodites were observed during the *R. hopkinsi* experiment.

At the highest concentration of powdered fish food there was 100% mortality for adult copepods (all species), and consequently no juvenile life stages were observed (Fig. 1d-f). Dissolved ammonia concentrations remained near or below 9 mg total ammonia/L (pH 7.9-8.1) during the tests and were unlikely to have been the cause of the low reproduction at higher powdered fish-food treatments. Previous studies have indicated that harpacticoid copepods are tolerant of low ammonia concentrations. For example, the 96-h LC_{50} values for total ammonia (NH_3 and NH_4^+) were reported to range from 14.6 to 18.2 mg/L for adults of five harpacticoid copepod species exposed to ammonia (Di Marzio et al. 2009). However, Linden et al. (1979) reported a much lower 96-h LC_{50} of 4.5 mg total NH_3/L for *N. spinipes*. LC_{50} experiments conducted in this study for adult *N. spinipes*, indicated that the lowest observed effect concentration (LOEC) for dissolved ammonia was >20 mg total ammonia/L. The considerable decrease in the dissolved oxygen concentration at the higher feeding rates of powdered fish food (Fig. 2) indicates that hypoxia could have caused the offspring reduction for the medium and high powdered fish food treatments.

For *N. spinipes*, increased amounts of algae as food stimulated offspring production and the rate of nauplii maturity, resulting in a positive relationship (Fig. 1). When powdered fish food was used, a significant increase ($p<0.01$) in the production of nauplii was seen in the low and medium treatments (which were not significantly different from each other ($p>0.01$))

355 when compared to controls. As for algae, the low fish food treatment increased the rate of
356 nauplii maturity, however the medium treatment resulted in a reduction in the rate of maturity
357 where a significantly lower number of copepodites were produced compared to the lower fish
358 food feeding regime ($p<0.01$). No organisms survived the seven days at the highest powdered
359 fish food concentration. The response of *N. spinipes* to the two diets was compared to
360 determine which feeding regime triggered the best response in terms of reproductive output. It
361 is clear from Fig. 1a that the addition of 1×10^7 cells/feed (high algae treatment) produced the
362 greatest number of nauplii and copepodites, which was not significantly different from the
363 result obtained from the addition of 0.15 mg of Sera® Micron per feed (low treatment) for
364 both nauplii and copepodites ($p>0.01$). While both of these diets induced a similar increase in
365 reproductive output of both *N. spinipes* and *R. hopkinsi*, the tri-algal diet was considered to be
366 superior as it did not result in a reduction in dissolved oxygen and is less likely to cause a
367 build up of ammonia in culture containers (Fig. 2). However, it remains untested whether an
368 algae concentration greater than 1×10^7 cells/feed could have produced better results.

369 *Tisbe tenuimana* responded best to the medium algae treatment, resulting in a higher
370 number of both nauplii and copepodites than the other algae treatments (including the
371 control), and a significantly higher ($p<0.01$) number of nauplii than any of the fish food
372 treatments. In addition, there was a significant ($p<0.01$) increase in the number of nauplii that
373 matured to copepodites. While feeding *T. tenuimana* the low fish food treatment resulted in
374 the highest number of copepodites of any treatment, the decrease in nauplii hatching made
375 this feeding regime less favourable compared to the reproductive response induced by other
376 feeding conditions. Based on the data presented in Fig. 1b, it is clear that the greatest
377 reproductive output was achieved by feeding 3×10^6 cells/feed.

378 For *Robertgurneya hopkinsi*, the treatments fed algae exhibited significantly greater
379 nauplii numbers than treatments with no added food ($p<0.01$). Increasing the amount of algae
380 did not increase the reproduction rate, instead it only resulted in a build up of excess algae in
381 the experimental vials. This may be attributed to the smaller size of *R. hopkinsi* (compared to
382 *N. spinipes*), or a lower need for food uptake as even the low algae treatment was sufficient to
383 sustain a high reproductive output for this species. In contrast, the addition of a powdered
384 fish food caused an increase in the number of nauplii hatching at the low treatment, but for the
385 medium and high fish-food treatments the number of nauplii produced decreased
386 significantly. Once again, this was attributed to reduced dissolved oxygen. When the number
387 of nauplii hatching in the low fish food treatment was compared to the algae treatments it

388 became evident that offspring production was significantly higher in the algae treatments
389 making the algal diet preferable for this species to promote reproductive output.

390 Our results suggest that the use of a tri-algal diet of *Tetraselmis* sp., *Isochrysis* sp. and
391 *P. tricornutum*: (a) is suitable to maintain laboratory cultures of *N. spinipes*, *R. hopkinsi* and
392 *T. tenuimana*; (b) promotes reproduction among adults of the copepod species tested; and (c)
393 is less likely to result in negative effects due to overfeeding, which was only observed in *T.*
394 *tenuimana*. The results also suggest that the substitution of a living algal diet with powdered
395 fish food is sufficient to maintain the health and reproduction of harpacticoid copepods if fed
396 at concentrations that do not result in a decrease in dissolved oxygen.

397 Recent studies have identified deleterious effects resulting from the ingestion of some
398 algae species by copepods (Lacoste et al. 2001; Dahl et al. 2009). This has been linked to the
399 presence of aldehydes in these organisms which inhibit egg hatching rates and recruitment in
400 copepods that graze on them (Miralto et al. 1999; Ianora et al. 2004). During our study we
401 became aware that Dahl et al. (2009) had observed that when *N. spinipes* was fed *P.*
402 *tricornutum*, lower reproduction and juvenile survival was observed compared to feeding with
403 the algae *Rodomonas salina*. These effects could potentially be caused by the presence of
404 aldehydes within the *P. tricornutum* cells, although this species has not been shown to possess
405 these chemicals. The results obtained from our study did not indicate that using *P.*
406 *tricornutum* within the tri-algal food mixture adversely affected the reproductive output of *N.*
407 *spinipes*, *T. tenuimana* or *R. hopkinsi*, shown by the comparable offspring production and
408 development observed between the algal and powdered fish food diets (Fig. 1). However,
409 because of potential deleterious effects from this algae species, it was omitted from the algal
410 mix fed during subsequent feeding of cultures and in sediment toxicity experiments.

411 *Sensitivity to dissolved copper*

412 The effect of dissolved copper on survival was assessed by exposing *N. spinipes* to
413 400, 600 and 800 µg Cu/L, *T. tenuimana* to 50 and 200 µg Cu/L, and *R. hopkinsi* to 200, 400,
414 600 and 800 µg Cu/L for 48 h. In general, it was observed that an increase in the
415 concentration of dissolved copper caused an increase in mortality for all three copepod
416 species used in this study (Fig. 3).

417 The time required to cause 50% lethality (LT_{50}) at the given dissolved copper
418 concentration was calculated to allow the sensitivity of the three species to be compared. *T.*
419 *tenuimana* was the most sensitive of the three species, with an LT_{50} value of 24 (22-27) h at

50 µg Cu/L. For *N. spinipes* the LT_{50} values for 200, and 400 µg Cu/L exposures were 114 (100-131) h and 36 (32-40) h, respectively. For *R. hopkinsi* the LT_{50} values for 200, 400, 600 and 800 µg Cu/L exposures were 119 (71-201) h, 25 (18-33) h, 10 (6-14) h and 7 (4-10) h, respectively.

Past studies have indicated that dissolved copper is ineffective as a toxicant to *N. spinipes* (Barnes and Stanbury 1948; Bengtsson 1978). Barnes and Stanbury (1948) found that a 24-h exposure to dissolved copper at a concentration of 260 µg/L caused 11.3% mortality in test organisms and a ten-fold increase in the copper concentration (to 2600 µg Cu/L) only caused 21% mortality. Bengtsson (1978) determined a 96-h LC_{50} of 1800 µg Cu/L. Both of these results greatly differ from the results obtained in the present study and may indicate that the *N. spinipes* isolated in NSW, Australia, is more sensitive to copper than the Bengtsson's isolates from the Gulf of Bothnia, Sweden.

To compare the sensitivity of these copepods to other test species, estimates were made of LC_{50} values. However, the data were not adequate for calculating an LC_{50} value for *T. tenuimana,* and the best point estimates calculated for the other species were for different exposure periods: 72-h LC_{50} value of 323 µg Cu/L for *N. spinipes*; and 96-h LC_{50} value of 238 µg Cu/L for *R. hopkinsi*. There was a strong correlation ($R^2 = 0.94$) between the 72-h survival of the species *R. hopkinsi* and *N. spinipes*, which indicated that their sensitivities to dissolved copper over a 72-h exposure period were very similar.

Selection of copepod species for routine toxicity tests

To permit an informed decision to be made about the most suitable copepod species for use in toxicity tests, a rank was generated which incorporated five parameters based on the results of our study in addition to general laboratory issues. Handling and culturing are important parameters when considering the suitability of a test species that is to be incorporated into routine laboratory test methods. This rank was influenced by the ease of handling, which considered catching and isolating individual copepods, which was dependant on the size and swimming speed of the species, and the ability to transfer the species between cultures and vials. Some species were more prone to being trapped on the surface of the water (due to surface tension) which increased the difficulty of handling the organisms. A rank was also provided for the ease of maintaining the copepod species in high density cultures under laboratory conditions, and the resilience of the population which could be estimated by the length of time cultures could be sustained before fouling occurred (due to the appearance and growth of nematodes, to which *T. tenuimana* were more susceptible). The

453 rate of species maturity was ranked based on observations in the laboratory where the
454 lifecycle of the species was determined, with species being ranked in order of shortest to
455 longest lifecycle. A shorter lifecycle was considered more desirable as it would lead to a
456 faster renewal of individuals in culture and is a desirable trait for test species of chronic life-
457 cycle based toxicity testing. The species response to added nutrition (by observing
458 reproduction and maturity rates) in the form of both algae and powdered fish food, and their
459 sensitivity to copper was also ranked.

460 Using rankings of 1 (most favourable), 2 or 3 (least favourable), the ranking for
461 handling, culturing, rate of maturity, food selectivity, and copper sensitivity, respectively,
462 were 2, 1, 1, 1, and 3 for *N. spinipes*, 1, 3, 2, 2, 3, and 1 for *T. tenuimana*, and 3, 2, 3, 2, and 2
463 for *R. hopkinsi***.** With a mean ranking of 1.6, *N. spinipes* was considered to be the most
464 suitable species for developing toxicity tests. This species was robust, easily cultured and was
465 reasonably sensitive to dissolved copper. While the sensitivity of *N. spinipes* to dissolved
466 copper was similar to that of *R. hopkinsi* (mean ranking = 2.4), the slower rate of maturity of
467 *R. hopkinsi* made it less desirable as a test species for chronic life-cycle based tests. Despite
468 being the most sensitive species to dissolved copper, *T. tenuimana* (mean ranking = 2.0) was
469 difficult to maintain in culture and did not respond well to the use of the powdered fish food.
470 The high sensitivity of *T. tenuimana* to dissolved copper deserves further investigation, as it is
471 possible that there are a large number of other harpacticoid copepods that may also be very
472 sensitive to contaminants, but not amenable to use in whole-sediment toxicity tests. *N.*
473 *spinipes* was selected as the species most suitable for future use in routine toxicity testing.

474 *Sensitivity of N. spinipes to dissolved contaminants*

475 A mean 96-h LC_{50} value for dissolved copper of 350±100 µg Cu/L (n=3) was
476 determined for adult *N. spinipes*. The sensitivity of other copepods to copper has been
477 determined for a range of exposure concentrations, e.g. 48-h LC_{50} of 256 µg/L for *Tisbe*
478 *battagliai* (Diz et al., 2009), 72-h LC_{50} of 450 µg/L for *Tigropus japonicus* (Kwok et al. 2008)
479 and 96-h LC_{50} of 150 µg/L for *Tigriopus brevicornis* (Barka et al. 2001). Amphipods are
480 commonly used for whole-sediment toxicity tests, and for comparison *Ampelisca abdita* has a
481 48-h LC_{50} of 30 µg Cu/L (Ho et al. 1999), while *M. plumulosa* a 96-h LC_{50} that decreases
482 from 470 µg Cu/L for 30-d old adults to 120 µg Cu/L the 5-d old juveniles (Spadaro et al.
483 2008). In general, *N. spinipes* exhibited sensitivity to copper in a range similar to these
484 species.

485 For Cd and Zn, no effects to survival were observed for concentrations up to 500
486 µg/L. *N. spinipes* was also not very sensitive to dissolved ammonia, with a 96-h LC_{50} value
487 of 300 mg total ammonia/L (pH 8). As ammonia occurs naturally in sediment pore waters,
488 when assessing the toxicity of sediment contamination it is often useful to use species that are
489 not highly sensitive to ammonia. For phenol, the 96-h LC_{50} for *N. spinipes* was 37 mg/L. For
490 comparison, 24-h LC_{50}s for phenol have been reported of 1.8 mg/L for the harpacticoid *T.*
491 *battagliai* (Smith et al. 1994) and 32 mg/L for the calanoid copepod *Acartia clause* (Buttino
492 1994).

493 *Use of N. spinipes for assessing sediment toxicity*

494 *N. spinipes* are iteroparous and females can produce multiple broods in the absence of
495 a male from stored reserves of sperm (Bengtsson 1978). When single culture-collected gravid
496 females were repeatedly separated from their released brood to new micro-well plates,
497 observations made two days after separation showed they were again gravid and more nauplii
498 had been released. The length of time this process could be repeated was not established, but
499 it continued for more than two weeks without the females encountering a male. Females
500 typically became gravid when food was abundant, and remained gravid for up to 48 h when
501 they drop their egg sack and the nauplii hatch. The development from nauplii to copepodites
502 was found to occur over 7-9 days and copepodites were observed from approximately day 9
503 to 20 of development before developing to mature copepods. After approximately 25 days,
504 gravid females began to be observed again.

505 Utilising the ability of *N. spinipes* to produce multiple broods over a short period of
506 time, the toxicity of sediments to *N. spinipes* was assessed by exposing gravid females to
507 sediments for 10 days. Although both nauplii hatching and some development to copepodites
508 occurred during this period, the endpoint used was the total reproductive output from gravid
509 females. The significance of the iteroparous behaviour of *N. spinipes* during the 10 days was
510 not expected to influence the interpretation of the results for different sediments. Along with
511 bacteria and algae present in the test sediments, additional food in the form of 2×10^4 cells/ml
512 of 1:1 *Isochrysis sp*. and *Tetraselmis sp*., as well as 0.3 mg Sera micron® fish food was
513 provided on days 2, 5 and 8. As dicussed earlier, *P. tricornutum* was not used due to the
514 potential deleterious effects from this algae species (Dahl et al. 2009). It was expected that
515 the powdered fish food would compliment the nutritional value of the algae.

516 The properties of the sediments tested and the reproductive output of *N. spinipes* is
517 shown in Table 1. The four control sediments had properties ranging from silty to sandy and
518 moderate to low TOC (Table 1), and the survival of gravid females was consistently >80%
519 and reproductive output of (mean±SE, range) of 35±7 (28-42) offspring/gravid. The
520 contaminated sediments caused significant reductions in the reproductive output (Table 1). A
521 combination of metals was likely to be the cause of the observed toxicity, with just 6
522 offspring/gravid (18% control) for a sediment that contained >2000 mg Zn/kg. For the
523 contaminated sediment C, it was believed that the high concentration of ammonia in the pore
524 waters (17 mg total ammonia/L, pH 8) was most likely responsible for the toxicity. For the
525 contaminated sediment D, it was believed that the 15 mg/kg cadmium concentration also
526 contributed to the observed toxicity.

527 **Conclusion**

528 Copepod cultures were successfully established in clean plastic containers with a 0.5–
529 1 cm layer of a mixture of silty sediment and clean sand (~50% particle size <63 µm) overlaid
530 with filtered seawater (30 PSU) of a depth of approximately 10 cm. The water was
531 continuously aerated and renewed weekly. Cultures and bioassays were successfully
532 maintained in a temperature controlled laboratory (21 ± 2 °C) under ambient light conditions.
533 The optimal feeding comprised of approximately 30 ml of a 1:1 mix of two algae species
534 (*Tetraselmis sp.* (CS-87), and *Isochrysis* sp. (CS-177), total concentration 1×10^7 cells/ml) as
535 well as 5 mg Sera micron® fish food (<63 µm) per culture twice a week, however, the
536 amounts of each algae could be varied if build up of one species was occurring (*Tetraselmis*
537 *sp.* is green and *Isochrysis* sp. is brown in colour). New copepod cultures were initiated every
538 2-3 weeks by transferring 100 gravid females from the existing cultures to new culture
539 containers.

540 Based on the ease of handling and culturing, rate of maturity, food selectivity and
541 sensitivity to copper, *N. spinipes* was determined to be the most suitable species for
542 developing sediment toxicity tests. The measurement of total reproductive output of *N.*
543 *spinipes* over 10-days was found to be a useful endpoint for assessing the effects of sediment
544 contamination. The test is relatively rapid, easy to perform using minimum sediment
545 volumes, the endpoint relatively easy to measure and appeared to be as sensitive to sediment
546 contaminants as other whole-sediment toxicity methods (McGee et al. 2004; Greenstein et al.
547 2008; Kennedy et al. 2009; Mann et al. 2009). It is likely that other test endpoints may also be

548 available using *N. spinipes*, including survival, gravidity, reproduction and development of
549 nauplii to copepodites.

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721 **Table 1** Sediment properties and effects to reproductive output of *N. spinipes* caused by
722 exposure to contaminated sediments.

	Controls				Contaminated				
Parameter	I	II	III	IV	A	B	C	D	E
Fines (%)	98	49	16	0.1	37	59	8	37	25
AVS (µmol/g)	5	2	40	0.1	0.4	<0.1	0.5	0.4	<1
TOC (%)	4.5	2.4	1.4	0.1	2.2	4.5	0.62	2.2	3.0
Fe (%)	2.4	1.2	0.58	0.06	0.96	3.7	0.16	0.96	2.2
Mn (mg/kg)	71	37	33	2.6	29	579	97	30	82
Cu (mg/kg)	25	13	64	1.0	10	835	35.2	37	126
Pb (mg/kg)	60	31.2	5	2.3	163	44.5	9.7	177	255
Zn (mg/kg)	216	109	120	1.2	437	401	145	431	2120
Total NH_3 (mg/L)	4.5	1.0	5	0.1	1	2	17	1	1
Reproduction, % control	120	119	92	106	59	51	44	33	18
Mean±SE	±17	±6	±20	±6	±8	±3	±4	±7	±11

723 Fines = particles of size <63 µm. AVS = acid-volatile sulfide. TOC = total organic carbon.
724 Cadmium concentrations were <1 mg/kg in all sediment except, D which had 15 mg Cd/kg.
725 Nickel concentrations ranged from 1-12 mg/kg in controls and 2-40 mg/kg in the contaminated sediments

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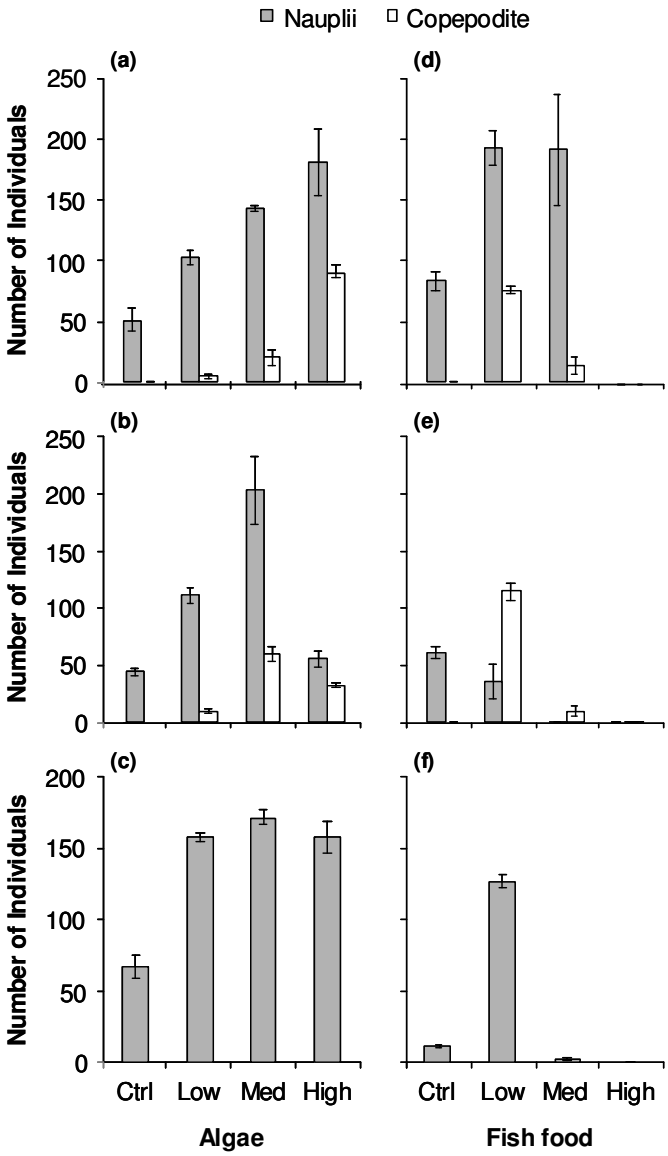

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729 Fig. 1. The reproductive output of *N. spinipes*, *T. tenuimana* and *R. hopkinsi* when fed a tri-
730 algal diet (a-c, respectively; at concentrations of 0, 1, 3 and 10 $\times 10^6$ cells per feed for control,
731 low, medium and high treatments, respectively) and powered fish food (d-f, respectively; at
732 concentrations of 0, 0.15, 0.5 and 1.5 mg per feed for control, low, medium and high
733 treatments, respectively).The number of nauplii (■) and copepodites (□) were counted
734 following 7 days of feeding at the respective diet and rate (mean ± standard error, n=4)

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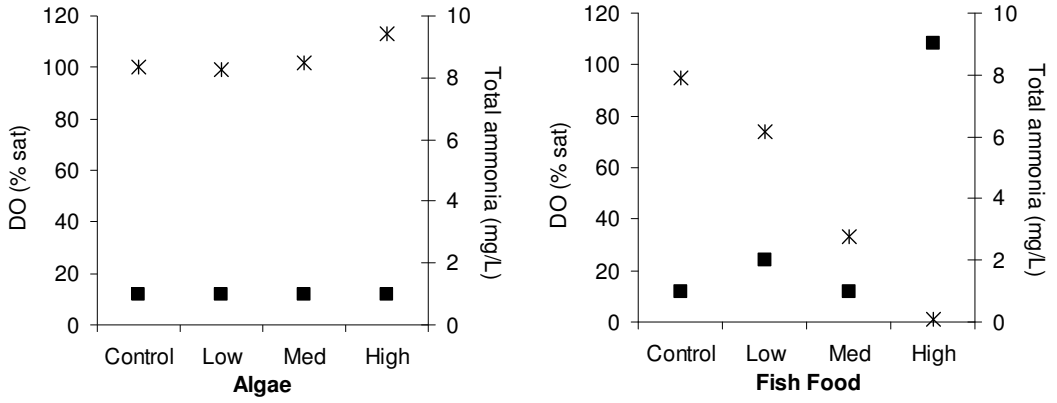

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738 Fig. 2. Dissolved oxygen (DO, % saturation, ∗) and ammonia (mg/L, ■) concentrations
739 measured in the test vials of the feeding experiment for *N. spinipes*

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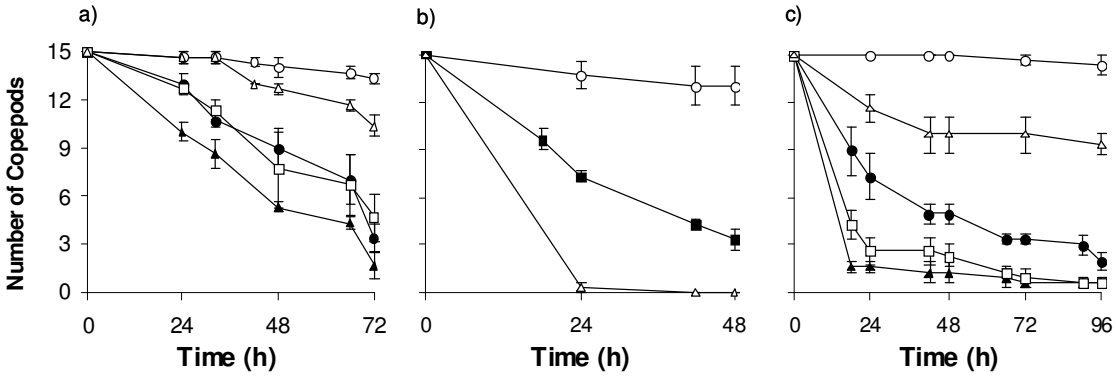

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744 Fig. 3. The survival of a) *N. spinipes*, b) *T. tenuimana* and c) *R. hopkinsi* (mean ± SE, n= 4)
745 when exposed to dissolved copper for ≥ 48 h. Nominal copper concentrations were 0 (○), 50
746 (■), 200 (▲), 400 (●), 600 (□) and 800 (▲) µg/L

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Supplementary Material

Plate 1. Photographs of (i) *Nitocra spinipes* (Boeck, 1864) (~24day life cycle), (ii) *Tisbe tenuimana* (Giesbrecht, 1902) (~28-day life cycle), and (iii) *Robertgurneya hopkinsi* (Lang, 1965) (~35-day life cycle). The scale bar represents a length of 250 µm.