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RESEARCH ARTICLE

The toxicity of arsenic(III), chromium(VI) and zinc to groundwater copepods

G. C. Hose[1,2] · K. Symington[1,2] · M. J. Lott[1] · M. J. Lategan[2,3]

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Abstract Groundwater ecosystems globally are threatened by anthropogenic contamination, yet there are few ecotoxicological data using obligate groundwater biota on which to base risk assessments. Copepods are found inhabiting aquifers of different geologies around the world and so are a useful taxon for use in ecotoxicological studies of groundwater. The aim of this study was to test the sensitivity of obligate groundwater copepods to metal contaminants (arsenic(III), chromium(VI) and zinc) in groundwater in static 96 h, 14 days and 28 days exposure tests. The copepods were variably sensitive to As, Cr and Zn, with Cr being the most toxic across all taxa. No taxon was consistently most sensitive and there was no apparent relationship between the hardness, pH and organic carbon concentration of the diluent water and the sensitivity of biota. As expected, toxicity increased with exposure period and we encourage the use of longer exposure periods in future toxicity tests with groundwater organisms to reflect the greater exposure periods likely to be associated with groundwater contamination.

Keywords Stygofauna · Stygobiont · Groundwater · Aquifer · Upland swamps

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✉ G. C. Hose
Grant.Hose@mq.edu.au

[1] Department of Biological Sciences, Macquarie University, NSW, Sydney 2109, Australia

[2] Cooperative Research Centre for Contamination and Remediation of the Environment (CRC CARE), Callaghan, Australia

[3] Department of Chemistry and Biomolecular Sciences, Macquarie University, NSW, Sydney 2109, Australia

Introduction

Groundwater ecosystems are emerging globally as a biodiverse resource (Humphreys 2006). These ecosystems are characterised by lack of light, low nutrient and carbon availability and relative climatic stability. In these conditions, an invertebrate fauna has evolved that contains a diversity of unique taxa not found in surface environments (Humphreys 2006).

Groundwater ecosystems tend to have a simple trophic structure and often lack vertebrates, with meiofaunal crustaceans often the highest order consumers (Humphreys 2006). In the stable groundwater environment, most animals have evolved classic K strategist traits of longevity and low metabolic and reproductive rates, as well as blindness and lack of body pigmentation in response to the dark, low-energy environment (Hose et al. 2015). Obligate groundwater fauna are therefore morphologically and physiologically different from related surface water taxa, and as a result, may be expected to respond differently to toxicants compared to surface-dwelling species (Hose 2007).

Contamination of groundwater resources is a global concern. Contamination by metals as a result of industrial wastes is unfortunately commonplace (Hashim et al. 2011). Not only does contamination threaten drinking water supplies but also the unique diversity of groundwater ecosystems. Despite this, the assessment of ecological risk of contamination of groundwater has been limited. There have been very few

ecotoxicological studies using groundwater fauna (see Avramov et al. 2013) and to date, most risk assessments have used surface water taxa as a surrogate (e.g. Baun et al. 1999; Gustavson et al. 2000; Daam et al. 2010; Crevecoeur et al. 2011), although this may not always be appropriate (Hose 2005).

Copepods are a common component of groundwater faunas (Galassi 2001). Harpacticoida and Cyclopoida are by far the most diverse and widespread inhabitants of groundwater among the Copepoda (Galassi 2001). The often isolated nature of groundwater ecosystems means that taxa colonising aquifers have diverged over time, giving rise to 'short-range endemic' species (sensu Harvey 2002), that is, taxa that are limited to a very narrow geographic range. Groundwater taxa separated by even several hundred metres may show strong genetic divergence (Hose 2008; Asmyhr et al. 2014). Such genetic divergence may afford variable sensitivities to toxicants so it is critical that a range of groundwater taxa are tested to better understand the relative sensitivities of groundwater copepods.

The geology of aquifers strongly influences the chemistry of the groundwater within it, such that water from different aquifers will have different ionic compositions, and so interact with toxicants in different ways (e.g. Hyne et al. 2005; Park et al. 2009). Indeed, the ionic composition of diluent waters is critical in affecting the response of biota to toxicants, particularly in hard waters such as are common in groundwater. Accordingly, the aim of this study was to test the sensitivity of groundwater copepods to contaminants (As, Zn and Cr) in groundwater. We compare the sensitivity of cyclopoid and harpacticoid copepods collected from different aquifer types in order to establish a preliminary understanding of the sensitivity of stygobiontic copepods to common metal and metalloid contaminants.

Materials and methods

Test species

Two species of cyclopoid and one species of harpacticoid copepods were tested. All taxa are previously undescribed and shared the typical traits of groundwater-adapted taxa (stygobionts) of having a lack of pigmentation and no eyespots. The first of the cyclopoid taxa (Cyclopoida: Cyclopidae) was collected from a groundwater-fed, upland peat swamp in the Budderoo National Park near Robertson NSW (34°37′S, 150°40′E, bore depth = 1.8 m, see Fryirs et al. 2014a, b and Hose et al. 2014 for description). Animals were collected using a bailer with which groundwater was withdrawn from a shallow piezometer. Ten litres of water was withdrawn from the piezometer and passed through a 63-μm-mesh sieve to collect the invertebrates. The invertebrates were placed in a sealable, 1-L plastic container which was filled with groundwater and placed in a portable cooler for transportation to the laboratory. In the laboratory, containers containing the invertebrates were placed in a dark environmental cabinet at 18 °C, which approximates the temperature of the groundwater at the time of collection.

The second cyclopoid taxon (Cyclopoida: Cyclopidae) and the harpacticoid taxon (Harpacticoida; Ameiridae) were collected from a fractured sandstone aquifer at Somersby, NSW Australia, approximately 80 km north of Sydney (33°22′S, 151°18′E, bore depth = 22 m). Animals were collected using methods as described in Korbel and Hose (2015) and as summarised below. Groundwater was pumped using a motorised inertia pump (Waterra, ON, Canada). Three hundred litres of water were removed and passed through a 63-μm-mesh sieve to collect the invertebrates. The invertebrates were placed in a sealable, 1-L plastic container which was filled with clean groundwater and placed in a portable cooler for transportation to the laboratory, where they were housed as described above. All animals were acclimated to conditions in the environmental cabinet for at least 48 h prior to testing. Tests were commenced within 7 days of collection. There was no evidence of organism mortality during this pretest period.

The taxonomy of copepod in Australia in general, groundwater in particular, is poorly known (Pesce et al. 1996). All specimens used in respective toxicity tests were morphologically similar under examination at ×60 magnification. In the absence of taxonomic keys below family level, we sequenced the cytochrome oxidase 1 gene for a number of specimens collected as described below.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from whole animals using the Bioline Genomic DNA extraction kit (Bioline, London, UK) as described in the manufacturer's protocols (Bioline, London, UK). Amplification of the mitochondrial cytochrome c oxidase subunit 1 (CO1) was accomplished using the primer set ZplankF1_t1 (5′-tgtaaaacgacggccagtTCTASWAA TCATAARGATATTGG-3′) and ZplankR1_t1 (5′-caggaaacagctatgacTTCAGGRTGRCCRAARAATCA-3′) (Prosser et al. 2013).

Polymerase chain reactions (PCRs) were performed in volumes of 25 μL, consisting of 12.5 μL Gotaq Green Mastermix, 0.2 μM of each primer, 9.5 μL PCR H_2O, and 2 μL of template DNA. An initial denaturation step of 95 °C for 1 min preceded 5 cycles of 94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min. This was followed by 35 cycles of 94 °C for 40 s, 51 °C for 40 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. PCR products were resolved by electrophoresis on 2 % agarose gels and purified using the QIAquick PCR Purification Kit according to the manufacturer's Spin

Protocol (Qiagen, Hilden, Germany). Sequencing was performed using the primers M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGCTATGAC3′) (Messing 1983) on a 3730 × L DNA analyser at the Macrogen sequencing facility (Macrogen Inc., Seoul, South Korea). All CO1 sequences generated in the course of this study have been deposited in GenBank.

CO1 sequences were examined for ambiguities and assembled into contigs using the program Geneious version 9.0.4 (Biomatters Ltd., Auckland, New Zealand). Alignment was by ClustalW employing the default parameters. The Kimura two-parameter (K2P) distance model was used to calculate nucleotide divergences within and between major copepod clades in MEGA 6.06 (Tamura et al. 2013).

Toxicity testing

Toxicity tests were conducted in untreated 24-well tissue culture plates. Animals were placed individually into wells using a micropipette with a known volume of diluent water. The number of individuals used in a test was dictated by the number collected. Tests with only a small number (≤35) of individuals were repeated and pooled in order to provide sufficient numbers for a robust toxicity estimate (for details see Online Resource 1). Steps toward validation of the method followed OECD (2005).

Tests were conducted using the groundwater from the site where the animals were collected as the diluent water. Treatment concentrations were randomly allocated to each well, and the required volume of clean groundwater water was added such that the subsequent addition of toxicant solution would give a final test volume of 2.0 mL. Each test was comprised of a negative control and five or seven test concentrations, each with four or three replicate wells per plate (respectively). The concentration ranges used for each toxicant were as follows: for As(III) 0–5000 mg As/L, Zn 0–100 mg Zn/L and Cr(VI) 0–1000 mg Cr/L. With consecutive experiments, the test concentrations were refined to provide a more accurate estimate of toxicity endpoints. By convention, results from a test were not accepted if there was more than 10 % mortality of organisms in control treatments (Connell et al. 1999).

Toxicity tests were conducted over 14 or 28 days with the test endpoint being mortality, defined as failure to move body or sensory appendages within 15 s of being gently prodded, as viewed under a microscope. Assessments of morbidity were conducted twice within approximately 30 min to provide certainty of mortality rather than dormancy/immobility. Mortality was recorded at 96 h and 14 and 28 days. Dissolved oxygen (HI9142, Hanna Instruments, Woonsocket, RI, USA), pH (HI9124, Hanna Instruments, Woonsocket, RI, USA), conductivity and temperature (TPS, Brendale, QLD Australia) were recorded every 48 or 72 h from a randomly selected replicate of each treatment using hand-held metres with associated small diameter probes.

Toxicant preparation and analysis

Toxicant stock solutions were made up in Milli-Q water. Chromium(VI) was added as $K_2Cr_2O_7$ (Sigma-Aldrich, 99 % purity) and arsenic was added as $NaAsO_2$ (Sigma-Aldrich, 98 % purity). Zinc was added as $ZnSO_4.7H_2O$ (Ajax Finechem, 99 % purity).

Nominal test concentrations were confirmed using total reflection X-ray fluorescence (TRX-RF). The method for aqueous samples is outlined in Klockenkamper and von Bohlen (1996); a 1 mL aliquot of sample solution, taken from replicates across the same plate, was spiked with gallium standard solution to give concentrations in the 0.1–100 mg/L, similar to the nominal toxicant concentration. The spiked gallium serves as an internal standard in the TRX-RF analysis (Klockenkamper and von Bohlen 1996). Aliquots of 10 μL of the spiked sample solution were then pipetted onto clean, hydrophobic carriers and dried before analysis. Samples from several randomly selected replicates per test were analysed. This method was also used to measure the background concentrations of metals in the untreated groundwater. Field measurements of pH, electrical conductivity, temperature and dissolved oxygen of the groundwater used as the diluent water were made with hand-held metres. The hardness of the diluent groundwater was measured using EDTA titration method 2340B (APHA 2005), and the total organic carbon (TOC) was measured using method 5310c (APHA 2005).

Statistical analysis

Measured and nominal test concentrations were compared using product moment regression in R version 3.0.3 (R Core Team 2014). Concentration response curves were estimated by fitting a 2-parameter, nonlinear regression assuming a binomial error structure for the mortality data. These analyses were done using the DRC package version 2.5-12 (Ritz and Streibig 2005) in R version 3.0.3 (R Core Team 2014). The model parameters were estimated using maximum likelihood, with starter values determined by the programs self-starter function. Effective concentration values (EC10 and EC50) were extrapolated from the fitted curve. A series of curves were fit to the data from each test; these include log-logistic, Weibull and log-normal curves. The best fitting model for each test was chosen by comparison of Akaikes information criterion. EC10 and EC50 values were compared between taxa using a t test of the difference in the mean (Wheeler et al. 2006) with significance level (α) at 0.05.

Where duplicate tests were conducted, concentration response curves from the separate tests at 96 and 48 h were compared using mixed-model nonlinear regression analysis

(Nielsen et al. 2004). In this approach, the full model (with response curves separated as random effects) and the simpler reduced model (with curves combined and so without random effects) were tested using a likelihood ratio test based on the residual deviance from each model (Nielsen et al. 2004).

Acute to chronic ratios (ACR) were estimated based on the acute (96 h) EC50 value and the corresponding chronic low effects (EC10) value. The chronic EC10 value was used here in lieu of a chronic no observed effect concentration (NOEC) value that is typically used (Hoff et al. 2010).

Results

Water quality

Measured test concentrations were close to nominal concentrations (nominal = 1.02 × measured, r^2 = 0.98, n = 74) so nominal concentrations were used in all subsequent analyses. Dissolved oxygen concentrations remained at 60 % saturation or higher throughout all tests (Online Resource 1). This level of oxygen saturation exceeds the concentrations generally observed in the field (Table 1). Temperature was consistently at or close to the test temperature of 18 °C. The hardness of the diluent water was relatively low (Table 1), which reflects the low carbonate concentrations in the sandstone at both collection locations. The pH of groundwater from both sites was similar and low (Table 1). The pH of the test solutions was similar over time within tests, similar between repeat tests (Online Resource 1) and similar to the water quality at the time of collection (Table 1).

Test species taxonomy

Molecular analysis supported our taxonomic assignments at the family-level, but available sequence databases provided little further detail. The analyses identified two distinct clades within the cyclopoids from Budderoo (Accession Nos KX244830-38) and a single clade within each of the cyclopoids (Accession numbers KX244839-41) and harpacticoids (Accession numbers KF361325, KF361326, KF361332) from Somersby. The mean Kimura-2-parameter pairwise divergence was greater than 25 % among the five major clades (Online Resource 2). Within-clade divergences were all below 2 % (Online resource 2).

Table 1 Physico-chemical variables and concentrations of common metals in the groundwater from each of the study sites

	Units	Budderoo	Somersby
pH		4.6–5.0	4.2–5.6
Conductivity	µS/cm	95	131–195
Dissolved oxygen	% Saturation	18–52	59–83
Hardness	mg/L as $CaCO_3$	8–37	25–44
Total organic carbon	mg/L	21–35	3–13
As	mg/L	<0.01	<0.01
Cr	mg/L	<0.01	0.03
Zn	mg/L	0.07	0.02

Toxicant response

Control mortality was less than 10 % (in most cases there was no control mortality) over 14–28 days. Any mortality that did occur in the controls typically occurred in the first 96 h (Table 2) which suggests that mortality may have been due to handling rather than to test conditions. Control mortality occurred after 96 h in only 1 of the 15 tests.

There was no significant difference in 96-h EC50 values for copepods exposed to As (Table 2). After 14 days, the EC50 values for the Somersby copepods were lower than that for the Budderoo cyclopoid but after 28 days, the difference between the cyclopoids was no longer significant. The EC10 values for the Budderoo cyclopoid were significantly higher than those for the Somersby copepods at all times (Table 2). The 14-day EC values were lower than the respective 96-h values across all taxa. There was a significant decrease in EC50 values between 14 and 28 days for the Somersby cyclopoids (Table 2), but not for the Budderoo cyclopoid nor the EC10 values.

The EC10 and EC50 values for the Somersby harpacticoid when exposed to Cr were significantly different to those of the two cyclopoid taxa copepods at all time points (Table 2). There was no significant difference in EC10 or EC50 values of the two cyclopoids at any time point (Table 2). There was a significant decrease in EC10 and EC50 values between 96 h and 14 days for the cyclopoids. The significant decrease in EC50 values continued for both cyclopoids between 14 and 28 days (Table 2). EC values for the harpacticoids were not significantly different over time as a consequence of large errors around those EC estimates (Table 2).

There were no significant differences in the EC10 values between taxa at any time point. The EC50 values for Zn suggest that the cyclopoid taxa were less sensitive to Zn than the harpacticoid taxon (Table 2). The EC50 values for the harpacticoid were significantly lower than those for the Somersby cyclopoids although not so for the Budderoo cyclopoid because of large errors associated with EC50 estimates for the Budderoo cyclopoid. Differences in EC10 and EC50 values between 14 and 28 days were significant for the Somersby cyclopoid but not so for the Budderoo cyclopoid (Table 2).

For all repeat tests, there was no significant difference in response curves at 14 days. However, there was a significant variability in response curves at 96 h for Cr and Zn. Acute to chronic ratios were calculated for each taxon and toxicant using the 96-h LC50 values and the 14- or 28-day LC10 value. The ACRs ranged from 4 to 374 (Table 3).

Table 2 Toxicity of As, Cr and Zn to groundwater copepods from different aquifers

Toxicant	ECx	Duration	Budderoo cyclopoid	Somersby cyclopoid	Somersby harpacticoid
As	EC10	96 h	9.50[a]	1.04[b]	0.32[b]
			(4.40–14.61; 105, 0/13)	(0.32–1.76; 185, 0/22)	(0–0.70; 76, 0/19)
		14 days	3.24[a]	0.08[b]	0.13[b]
			(1.98–4.51; 105, 0/13)	(0.002–0.163; 185, 0/22)	(0–0.30; 76, 0/19)
		28 days	2.67[a]	0.04[b]	-
			(0.21–5.12; 105, 0/13)	(0.000–0.070; 157, 0/17)	
	EC50	96 h	10.72[a]	5.24[a]	3.04[a]
			(2.89–18.55; 105, 0/13)	(2.99–7.49; 185, 0/22)	(0.20–5.88; 76, 0/19)
		14 days	5.57[a]	0.79[b]	1.46[b]
			(3.90–7.25; 105, 0/13)	(0.45–1.14; 185, 0/22)	(0.45–2.47; 76, 0/19)
		28 days	3.63[a]	0.25[a]	-
			(0–8.92; 105, 0/13)	(0.13–0.38; 157, 0/17)	
Cr	EC10	96 h	>1*	0.75[a]	0.037[b]
			(–; 121, 0/19)	(0.25–1.26; 184, 2/38)	(0–0.078; 84, 0/25)
		14 days	0.12[a]	0.12[a]	0.002[b]
			(0.04–0.20; 121,1/19)	(0.02–0.23; 184, 2/38)	(0–0.007; 84, 0/25)
		28 days	0.08[a]	0.02[ab]	0.002[b]
			(0.03–0.12; 121, 1/19)	(0–0.06; 53, 1/13)	(0–0.007; 84, 0/25)
	EC50	96 h	>1*	3.08[a]	0.70[b]
			(–; 121, 0/19)	(1.85–4.31; 184, 2/38)	(0–1.70; 84, 0/25)
		14 days	0.54[a]	1.06[a]	0.03[b]
			(0.34–0.74; 121, 1/19)	(0.58–1.55; 184, 2/38)	(0–0.07; 84, 0/25)
		28 days	0.27[a]	0.22[a]	0.02[b]
			(0.19–0.35; 121, 1/19)	(0.05–0.40; 53, 1/13)	(0.00–0.05; 84, 0/25)
Zn	EC10	96 h	6.04[a]	1.24[a]	0.34[a]
			(0–33.97; 74, 0/9)	(0.26–2.23; 118, 0/19)	(0.13–0.54; 107; 1/29)
		14 days	0.06[a]	0.43[a]	0.28[a]
			(0–0.18; 74, 0/9)	(0.05–0.81; 118, 0/19)	(0.02–0.55; 107; 1/29)
		28 days	0.03[a]	0.01[a]	-
			(0–0.07; 74, 0/9)	(0.0–0.03; 28, 0/11)	
	EC50	96 h	>10*	6.25[a]	0.88[b]
			(–; 74, 0/9)	(3.42–9.08; 118, 0/19)	(0.60–1.15; 107; 1/29)
		14 days	2.42[ab]	3.13[a]	0.74[b]
			(0.12–4.72; 74, 0/9)	(1.69–4.58; 118, 0/19)	(0.51–0.96; 107; 1/29)
		28 days	0.77[a]	0.50[a]	-
			(0.17–1.37; 74, 0/9)	(0.11–0.90; 83, 0/11)	

EC10 and EC50 values indicate the concentration (mg/L) effecting 10 and 50 % of the test population, respectively. *Values in parentheses* indicate 95 % fiducial limits, rounded up to 0 for lower values, followed by sample size (*n*) and control mortality expressed as number dead/number of control animals. *Different superscript letters* denote significant differences ($p < 0.05$) in ECx values among taxa (i.e. values in each row)

*EC estimate exceeds highest test concentration

Discussion

Performance of the tests

Toxicity testing with groundwater fauna provides a number of challenges, not least of which is the collection of sufficient numbers of organisms for testing. As a consequence, we were required to pool data from tests using animals collected on different occasions. Fortunately, the response curves for these taxa were not significantly different after 14-day exposure although there was some significant variability between response curves after 96 h.

With the uncertainty of finding groundwater animals in field collections, pooling of data over space or time is potentially the only means of achieving sufficient animals for a robust test. However, given that groundwater fauna often

Table 3 Acute to chronic ratios for each toxicant and copepod taxon, based on ratio of 96-h EC50 and 14–28-day EC10 values

Toxicant	Budderoo cyclopoid	Somersby cyclopoid	Somersby harpacticoid	Geometric mean ACR
As	4	66	374[a]	46
Cr	>13	154	350	88
Zn	>333	15	6[a]	31

Greater than sign indicates highest test concentration used in lieu of 96-h EC50

[a] 14-day EC10 used as chronic value

show a high degree of endemism over small spatial scales (e.g. Harvey 2002), collecting animals from a single location over time (rather than multiple locations at one time) is the most suitable approach to ensure that the same organisms are being collected. The trade-off of repeated collections is that it may take some time to accumulate sufficient response data to determine robust ECx values using this approach. Unfortunately, the low fecundity and slow growth of stygofauna make laboratory culture of large numbers of animals (as needed for toxicity testing) particularly difficult (Thulin and Hahn 2008; Avramov et al. 2013).

It is a further challenge that the majority of stygofauna (at least in Australia) are undescribed (Guzik et al. 2010). In this study, we used morphological traits to identify the specimens tested which led us to expect a single taxon was present within each location. In lieu of relevant taxonomic keys beyond the family level, we used analysis of the CO1 'barcoding' gene for provide more detailed taxonomy. This analysis provided two unexpected outcomes. Firstly, we learnt little more of the taxonomy of the test taxa because there was little homology between sequences from our study taxa and those in existing nucleotide databases. Secondly, the single morphotype from Budderoo comprised two phylogenetic clades. The Kimura-2-parameter pairwise divergence was greater than 25 % for all five major clades, including between the two Budderoo cyclopoid clades. Divergence of >17 % is considered to reflect species level differences among Crustacea when using the CO1 gene (Costa et al., 2007). However, this high diversity that we have shown is perhaps not surprising given that cryptic species are relatively common within the Copepoda (Lajus et al. 2015). Within-clade divergences were below 2 %, indicating that each of the five major clades constituted a single copepod species.

The presence of two distinct clades with the Budderoo cyclopoids highlights a great challenge of using field-collected specimens for toxicity testing. Using field-collected animals ensures that relevant, endemic taxa are tested, and ensures genetic diversity among the test population, which are limitations of using laboratory-reared populations (e.g. Nowak et al. 2007). The trade-off is that taxonomic uncertainty is high because close examination of field-collected animals is avoided to minimise stress and handling before testing. The use of laboratory-reared taxa provides taxonomic certainty and also allows for standardisation of age or life stage in the test population, which likely serve to reduce the variability within and between tests that might be expected from mixed populations. We are fortunate that specimens from both Budderoo cyclopoid clades were present in all test populations that we analysed. Fortunately, the potential for greater variability with mixed populations appears to not have been a problem in this study, given that the variation within and between tests was similar to the tests for the Somersby taxa which we know to be of a single clade.

Physico-chemical conditions remained relatively constant during the tests and between tests with diluent water from the same source. Dissolved oxygen concentrations remained relatively high during tests, largely because of the small test volume and large surface area for gas exchange, meaning that dissolved oxygen concentrations during the test were somewhat higher than those typically recorded in groundwater (Table 1; Hahn 2006). It is our experience that these stygobitic copepods and other groundwater crustaceans (Amphipoda, Syncarida) survive well in oxygenated laboratory environments. As yet we have no data on the relative sensitivities of metals to fauna in normoxic and hypoxic environments; however, Notenboom et al. (1992), showed that dissolved oxygen concentrations (0.1–10 mg O_2/L) did not affect the toxic response of groundwater crustaceans to metals.

Control mortality was low (generally 0 %) and water quality conditions were stable during all tests, suggesting we had established a suitable testing environment. Further, the reproducibility of toxicity estimates from repeat tests at 14 days suggests that we have established a repeatable protocol for long-exposure tests. We acknowledge the variability in responses from acute (96-h) exposures and discuss below the significance of this for risk assessments in groundwater.

Toxicity of test compounds

The sensitivity of groundwater copepods to common metals was highly variable, spanning orders of magnitude between toxicants and copepod taxa. Such variation among toxicants has been recorded across several orders of subterranean taxa (e.g. Meinel et al. 1988; Canivet et al. 2001; Di Marzio et al. 2009). Importantly, neither of the three taxa tested was consistently the most sensitive across toxicants, suggesting that multiple taxa should be tested in order adequately assess ecological risk.

The acute toxicity of As varied between the tested taxa. The Budderoo cyclopoid had sensitivity to As that was similar to that of the stygobitic amphipod *Niphargus rhenorhodanensis* (240-h LC50, 3.97 mg/L; Canivet et al. 2001), which was the most tolerant of a range of taxa in that study, but the Somersby copepods were more sensitive. We could find no relevant acute exposure data for groundwater fauna to As (III), but note that the copepods tested here were much more sensitive than a groundwater fungi (96-h IC25 > 1025 mg/L; Lategan and Hose 2014). Overall, the copepods were generally less sensitive to As than Cr, which is consistent with the findings of Canivet et al. (2001).

The acute toxicity of chromium to the copepods was similar to that recorded elsewhere. Di Marzio et al. (2009) report 96-h LC50 values to a number of hyporheic harpacticoid copepods, including the stygophilous *Bryocamptus echinatus* exposed to hexavalent chromium; the range of 96-h LC50 values for those copepods (1.26–3.82 mg Cr/L) was similar to the 96-h EC50 value reported here for harpacticoid and cyclopoid copepods (0.70–3.08 mg Cr/L). Further, the range of acute EC50 values for the hypogean copepods tested in this study was similar to that derived for the *N. rhenorhodanensis* (1.51–2.07 mg Cr/L; Canivet et al. 2001) and within the range reported for the stygobitic isopod *Proassellus* spp. (48-h EC50 0.396–6.35 mg Cr/L; Reboleira et al. 2013) but lower than that for surface-dwelling marine and freshwater copepods from elsewhere (5.9–10.5 mg Cr L^{-1}; Baudouin and Scoppa 1974; Abbasi et al. 1988; Hutchinson et al., 1994). Unfortunately, there were few data with which to compare our 14- and 28-day exposure results, except that our 14-day EC50 values (0.54–1.06 mg Cr/L) for cyclopoids were similar to a 10-day LC50 for *N. rhenorhodanensis* (0.23 mg Cr/L; Canivet et al. 2001) and less than those for a range of epigean taxa (Ewell et al. 1986; Canivet et al. 2001). The 14-day EC50 value for Harpacticoida (0.03 mg Cr/L) was lower than similar duration L(E)C50 values reported for other micro-crustaceans reported above.

The EC50 values for the cyclopoid copepods exposed to Zn were similar across all exposure periods and were greater than those for the harpacticoid for which most mortality occurred in the first 96 h and changed little thereafter. The range of 96-h EC50 values for the Somersby copepods (0.88–6.25 mg Zn/L) spanned that determined by Notenboom et al. (1992) for the groundwater copepod *Parastenocaris germanica* (96-h LC50 = 1.7 mg Zn/L) under oxic (10 mg O_2/L) conditions and were well below those determined for groundwater crustaceans *Asellus cavaticus* (Isopoda) and *Niphargus aquilex* (Amphipoda) (90–180 mg Zn/L, Meinel et al. 1988). The 14-day LC50 values were also well below those previously reported for surface water crustaceans (e.g. 14-day LC50 84 mg Zn/L; Mirenda 1986).

Current approaches for risk assessment include the use of standard test species, such as *Daphnia magna* (e.g. Daam et al. 2010), as a first tier in lieu of groundwater biota. The groundwater copepods tested here were less acutely sensitive to zinc than *D. magna* (96-h LC50 0.15–0.18 mg Zn/L; Ewell et al. 1986, Lazorchak et al. 2009). The 96-h LC50 values for *D. magna* exposed to Cr (0.07–0.16 mg Cr/L, Fargasova 1994, Ewell et al. 1986) and 14-day LC50 value (0.006 mg/L) were approximately an order of magnitude lower than those for all of the copepods tested here (Table 1). The 96-h EC50 values for *D. magna* of 1.50–4.34 mg As(III)/L (Lima et al. 1984) were similar to those of the Somersby copepods (3.04–5.24 mg As(III)/L) but well below that of the Budderoo cyclopoid. The 14-day EC50 value for *D. magna* of 2.85 mg As(III)/L (Biesinger and Christensen 1972) was below the corresponding values for the Budderoo cyclopoid and above those for the Somersby taxa. Overall, these data suggest generally little congruence between acute (96 h) *D. magna* response data and the response of the groundwater copepods tested here.

The duration of tests for groundwater taxa is of critical importance to their environmental relevance. It is arguable that short-term exposures may not reflect groundwater conditions, with the exception of perhaps karst systems (see Reboleira et al. 2013), because of the slow movement of groundwater in most situations. It is usual for toxicity to increase and hence EC values to decrease with increasing exposure period and this trend was evident in this study, with decreases in EC values of up to two orders of magnitude between 96 h and 14 days, with further significant decreases from 14 to 28 days in some tests. Furthermore, there was considerable variability in ECx values between repeat tests at 96 h, but this variability decreased over time such that repeat tests were not significantly different after 14 days. Our evidence suggests that tests of 14 days or greater are most suitable for groundwater assessments; however, time-independent EC values (e.g. Avramov et al. 2013) would be particularly useful for applying in this situation and should be a direction of future research to reconcile the issue of long exposure times in groundwater.

As suggested by Hose (2007), the lower metabolic rate of hypogean taxa (Di Lorenzo et al. 2015) may mean that the uptake of toxicants occurs more slowly than it does in epigean taxa (Plénet 1999), particularly for toxicants that are actively taken up by the organism and for which the rate of uptake is metabolically dependent. As a consequence, acute exposures may greatly underestimate impacts to biota. However, a low metabolic rate also means that an organism's capacity to sequester, detoxify or excrete toxins by active pathways (Rainbow 2007) may also be limited. For toxins that are taken up passively, such as metals (Rainbow and Dallinger 1993), the uptake may exceed the low metabolic capacity of hypogean organisms for depuration or immobilisation, (such as the costly production of metallothionein protiens (e.g. Barber et al. 1990)), thus making them potentially more sensitive than

epigean species. Indeed, this appears to be the case for Cr and Zn in this study.

The organic rich environment of the Budderoo peat swamp (Hose et al. 2014), and subsequently higher dissolved organic matter concentrations in the test water (Table 1), potentially provided some protection for the copepods. The 14-day EC50 values for As were significantly higher for the Budderoo cyclopoid than for either Somersby copepod, which is consistent with the expectation that the toxicity of As is reduced in the presence of organic matter organic matter due to As binding to the organic matter and reducing the bioavailability (e.g. Burton et al. 1987). However, the same trend might also be expected for Zn (Paulauskis and Winner 1988) but was not observed. It is possible that in the low pH Budderoo groundwater (pH < 5.5), the influence of organic matter sorption on reducing bioavailability of Zn may be limited (Kalbitz and Wennrich 1998; Gundersen and Steinnes 2003), which may explain the lack of amelioration of toxicity in the organic rich water. The lack of difference in Cr toxicity to cyclopoids in Budderoo and Somersby diluent water is consistent with the findings of Park et al. (2009) who showed that dissolved organic matter did not ameliorate Cr toxicity.

Implications for risk assessment of groundwater ecosystems

The current dearth of toxicity data for groundwater organisms limits the development of robust risk assessments for GW ecosystems. Our experimental approach allows the future development of data for groundwater fauna and has been used successfully with other groundwater crustaceans (Hose, unpublished data). In addition to novel methods for testing elements of the groundwater microbiome (Lategan and Hose 2014; Lategan et al. 2016), we are progressing toward a point at which toxicity data may soon be available for a sufficient range of taxa to create an ecosystem-specific species sensitivity distribution for some toxicants, with the result from this of ecosystem-specific water quality criteria.

Until such time as ecosystem-specific criteria are available, water quality criteria for groundwater ecosystems remain limited to those generated for surface waters, or with specific criteria often based on analytical detection limits (such as 0.1 μg/L for pesticides in the EU, see Daam et al. 2010). The current Australian freshwater quality criteria for As(III) (0.024 mg/L) and Cr(VI) (0.001 mg/L) were similar to and just below the chronic EC10 values determined in this study. For Zn, the current Australian freshwater quality criterion of 0.008 mg/L (ANZECC and ARMCANZ 2000) is well below the concentrations causing mortality here and so is likely to be protective of the copepods tested. Considering these values, it is apparent that water quality criteria for metals in surface waters may not always be protective of groundwater systems.

While we recommend long exposure periods for toxicity testing with groundwater biota, the use of acute toxicity data for ecological risk assessments remains common practice, usually with the application of an ACR as a conversion factor to estimate chronic effects. The default ACR value in many risk assessment frameworks is 10 (e.g. ANZECC and ARMCANZ 2000; Hoff et al. 2010) for converting acute LC50 values to chronic no (or low) effects values. Based on our results, the ACR for all toxicants and taxa varied from 4 to 374, and 5 of the 9 ACR values greatly exceeded the default value of 10 (Table 3). The geometric mean ACR values for each toxicant were also well above the default value. The ACR values estimated here are similar to those expected for metals (1.1–434) and within the very broad range reported for freshwater invertebrates (1.1–18,550) (Raimondo et al. 2007). However, these results do highlight the need for ecosystem-specific assessments of ecological risk and that the processes of risk assessment for surface water systems should not be blindly applied to groundwaters.

In the development of this test, we have taken preliminary steps toward (pre)validation (Hartung et al., 2004; OECD 2005). The need for groundwater-specific toxicity tests is well established (Hose 2005, 2007; Daam et al. 2010), and the relevance of the chosen endpoint (mortality) has clear ecological relevance. We have demonstrated that across four toxicants (which may serve as reference toxicants), the temporal repeatability of the method was high, with there being no significant difference in the 14-day response curves between repeat tests. Although the coefficients of variation between 14-day EC50 values from repeat tests were somewhat higher than those reported in other validation studies (e.g. Lazorchak et al. 2009), this is likely to be addressed as the standard deviation is reduced with further repeat testing. Further, we have shown by comparison to literature values that the sensitivity of these groundwater taxa is different that of *D. magna* across different toxicants, which is expected given the likely physiological and behavioural differences between species. Control mortality was consistently low in all tests and below the criterion of 10 % for test acceptability. Further steps in validating this test will be to repeat these tests to examine longer-term variability and to undertake inter-laboratory comparisons, but the latter is somewhat complicated by the fact that these and other groundwater organisms are difficult to culture (Avramov et al. 2013) and that collections from different locations would yield different taxa which could confound comparisons.

Conclusions

The groundwater copepods were variably sensitive to As, Zn and Cr, with Cr being the most toxic across all taxa. There was neither clear relationship between sensitivity and copepod

taxon nor did water quality differences among aquifers relate consistently to the sensitivity of biota. As expected, toxicity increased with exposure period. Our results suggest that toxicity tests greater than 96 h duration are needed for groundwater biota and encourage the use of longer exposure periods in future toxicity tests.

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Conflict of interest The authors declare that they have no conflict of interest.

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