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Evaluation of a Methodology for Toxicity Testing of Volatile Chlorinated Hydrocarbons on Marine Organisms

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Abstract This study evaluated the suitability of sealed containers for toxicity testing to prevent loss of volatile chlorinated hydrocarbons (VCHs) with a range of Australian marine organisms including: micro-algae; sea urchin and oyster larvae in 44 mL sealed vials and fish larvae; amphipods; and juvenile polychaetes in 1 L sealed jars. Vials prevented volatilisation of VCHs during testing. Jars were less effective, with average losses of 46%. Growth and development of algae, sea urchins and oysters in vials was acceptable, indicating suitability of the methodology. Jars were suitable for amphipods and polychaetes; however, further evaluation of the fish test is required.

Keywords Volatile chlorinated hydrocarbons · Toxicity testing · Sealed containers

Penrhyn Estuary, in Sydney, Australia, receives groundwater contaminated with volatile chlorinated hydrocarbons (VCHs) and comparison of measured concentrations of VCHs in estuarine waters against the numerical limits (termed trigger values – TVs) for these chemicals in the Australian water quality guidelines (ANZECC and ARMCANZ 2000) indicated that VCHs posed an unacceptable hazard and that direct toxicity assessment (DTA) was warranted (Hunt et al. 2007). However, TVs for VCHs are classed as low reliability – meaning the amount and type of toxicity data on which they are based are not optimal. The Australian water quality guidelines (ANZECC and ARMCANZ 2000) identified generating additional toxicity data for chemicals with low reliability TVs as a key research priority. For both these reasons it is necessary to conduct toxicity tests where indigenous species are exposed to VCHs.

VCHs are characterised by high vapour pressures and Henry's Law Constants and are readily lost from open containers. As such, standard test protocols are not suitable, as volatilisation of VCHs would result in decreased exposure concentrations and underestimation of the toxicity. Recent work by Tsai and Chen (2007) indicated that toxicity testing for volatile narcotic contaminants undertaken in open containers underestimated toxicity to algae, when compared to testing in closed systems, by up two orders of magnitude, regardless of Henry's Law Constants. Although studies have been undertaken to develop test protocols for sealed test vessels, these have focussed on micro-algae (Brack et al. 1998; Galassi and Vighi 1981; Herman et al. 1990; Lin et al. 2005; Mayer et al. 2000), with some assessment of the suitability of sealed test vessels for cladocerans (Rose et al. 1997). Limited assessment of the suitability of these methods has been undertaken for other test organisms. This study presents a methodology for determining the toxicity of VCHs in sealed vessels for six indigenous marine organisms including: a sea urchin

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(*Heliocidaris tuberculata*); an oyster (*Saccostrea commercialis*); a micro-alga (*Nitzschia closterium*); a fish (*Macquaria novemaculeata*); an amphipod (*Allorchestes compressa*) and a polychaete (*Diopatra dentata*). Development of this methodology was done to support DTA of VCH contamination and evaluation of the appropriateness of the ANZECC and ARMCANZ (2000) trigger values for VCHs. Three contaminant treatments were evaluated; a complex mixture of 14 VCHs in groundwater from an industrial facility, which is the source of discharge to Penrhyn Estuary, and individual seawater samples spiked with 1,2-dichloroethane and chloroform.

The objectives of the current study were to determine: the suitability of jars (1 L) and vials (44 mL) in preventing the loss of VCHs during toxicity testing; and the suitability for toxicity testing with six indigenous Australian marine species.

Materials and Methods

The suitability of the sealed test vessels for toxicity testing using six indigenous marine species was evaluated by assessing the survival in negative controls of artificial sea water (ASW) and filtered seawater (FSW). Tests for small organisms, namely micro-alga, and larvae of the sea urchin and oyster were undertaken in 44 mL clear glass vials with Teflon^{TM} lined lids and no headspace. Toxicity tests with medium sized organisms, namely fish, amphipods and polychaetes were undertaken in 1 L glass jars sealed with Teflon^{TM}-lined lids containing approximately 500 mL water and 500 mL of headspace. Headspace was left in the jars to provide sufficient oxygen for the organisms. Toxicity test conditions for each of the test organisms are summarised in Table 1. For each test, temperature, pH, salinity and dissolved oxygen content of a sample from each treatment were measured at the start; immediately prior to renewal of test water; and at the conclusion of the test. Reference toxicants were undertaken for all tests with the exception of the juvenile polychaete worms (*D. dentata*), which had not previously been used as a test organism, and larval fish (*M. novemaculeata* – Australian Bass), to reduce the total number of organisms used in testing in accordance with the requirements of ethics approval granted for the project.

The first treatment was a complex mixture of VCHs obtained from contaminated groundwater at an industrial facility in Sydney, Australia. The groundwater sample was derived from two sources: shallow groundwater discharge collected from a stormwater drain and a groundwater sample from a nearby bundled piezometer. These samples were combined in a ratio of 9:1 (drain: piezometer), resulting in a concentration of approximately 100 mg/L of total VCHs. This manipulation was undertaken to ensure sufficiently high VCH concentrations to cause a response for all six test organisms. VCHs identified in the groundwater included: chloroform, vinyl chloride and tetrachloroethene; with 1,2-dichloroethane accounting for approximately 90% of the contaminant load by weight (Hunt et al. 2007). Seven serial dilutions of the groundwater sample were made, using FSW, in twofold dilutions, with the highest and lowest proportions of groundwater being 50% and 0.75%, respectively. The salinity of the groundwater was adjusted to marine conditions (approximately 30 ppt) using artificial sea salts. Negative controls were included for artificial seawater (ASW; to a maximum proportion of 50%) and FSW. The second and third treatments consisted of clean FSW, individually spiked with 1,2-dichloroethane and chloroform. These two contaminants were selected as their concentrations in groundwater exceeded the ANZECC and ARMCANZ (2000) 95% TVs in estuarine waters (Hunt et al. 2007). Clean seawater was collected from Lurline Bay, a coastal site near Sydney and filtered to 0.45 μm. Chloroform and 1,2-dichloroethane were purchased from Lab Scan Analytical Services (AR Grade, 99.8% purity). For each chemical, a stock solution was prepared in seawater, the nominal concentrations of which were 1,000 and 300 mg/L for 1,2-dichloroethane and chloroform, respectively. Each stock solution was serially diluted with seawater six times, by a factor of 3.

Samples were collected from test vessels to determine the potential loss of VCHs and inaccuracies during preparation of test solutions and during toxicity testing. To evaluate the loss of VCHs during preparation of test solutions, predicted (nominal) concentrations were compared to measured concentrations at the start of toxicity testing. Loss of VCHs during toxicity testing was evaluated by comparing measured concentrations of samples that were collected at the start ($t = 0$ h) and immediately prior to the renewal point ($t = 48$ h) of tests in jars, and at the start ($t = 0$ h) and end ($t = 72$ h) of tests in vials. To measure concentrations at the end of testing, an additional replicate vessel was prepared for each dilution. The vessel was filled with test solution and included in the incubator without test organisms. These samples represent the exposure concentration of organisms in the test vessels at the conclusion of testing (Table 2). Samples were collected and analysed from four of the seven dilutions in vials and all four treatments in jars (Table 2). Samples were collected in 40 mL glass vials with airtight Teflon^{TM}-lined lids with zero headspace, immediately preserved with hydrochloric acid and stored at <4°C. Samples were extracted using purge and trap methodology (USEPA 5030B) and analysed by gas chromatography and mass spectrometry (GC/MS) utilising a modification of USEPA

Table 1 Summary of toxicity test conditions for six test organisms

Test species	Sea urchin *Heliocidaris tuberculata*	Rock oyster *Saccostrea commercialis*	Alga *Nitzschia closterium*	Australian Bass *Macquaria novemaculeata*	Polychaete *Diopatra dentata*	Amphipod *Allorchestes compressa*
Test type	Static non-renewal	Static non-renewal	Static non-renewal	Semi-static renewal at 48 h	Semi-static renewal at 48 h	Semi-static renewal at 48 h
Test type[a]	Sub-chronic	Sub-chronic	Chronic	Acute	Acute	Acute
Test duration	72 h	72 h	72 h	96 h	96 h	96 h
Test end-point	Normal pluteus larvae	Larval development to D-veliger stage	Cell yield at 72 h	Imbalance, including survival	Survival	Survival
Test temperature	20 ± 1°C	20 ± 1°C	20 ± 1°C	20 ± 1°C	20 ± 1°C	20 ± 1°C
Test salinity	35 ± 1‰	35 ± 1‰	35 ± 1‰	35 ± 1‰	35 ± 1‰	35 ± 1‰
Test chamber	44 mL vial	44 mL vial	44 mL vial	1 L jar	1 L jar	1 L jar
Dissolved oxygen content (% saturation)	100.9–115.9	100.9–115.9	100.9–107.4	102.9–119.6	96.9–104.3	96.9–104.3
pH	7.6–8.3	7.6–8.3	7.6–8.3	7.5–8.1	7.7–8.1	7.7–8.1
Reference toxicant limits	7.5–10.1 µg Cu^{2+}/L	15.1–26.8 µg Cu^{2+}/L	19–24 µg Cu^{2+}/L[a]	Not Applicable	Not Applicable	0.84–5.4 mg NaDS[b]/L
Source of test organisms	Field collected, Sydney	Hatchery reared	CSIRO Marine algal supply service	Hatchery reared	Hatchery reared	Field collected, Portarlington

[a] Range identified in Hogan et al. (2005)

[b] NaDS (sodium dodecyl sulfate)

Method 8260B for volatile organic compounds (USEPA 1996). The two modifications were a reduction in the number of analytes and surrogates, given the known contaminants in the groundwater and analytes were quantified by a single point calibration after validation against a compliant five point calibration. The modified method has been approved the National Association of Testing Authorities (Australia). The limit of reporting was 1 µg/L for all analytes with the exception of vinyl chloride (10 µg/L). Quality control evaluations indicated that no analytes were detected in method blanks and recoveries for laboratory control samples and matrix spikes and differences between primary and duplicate samples were within accepted criteria.

Relationships between initial nominal and measured exposure concentrations were derived using simple linear and polynomial regression analyses. Geometric means of the measured concentrations at the start and end of each toxicity test for each treatment were used as the measured exposure concentrations. Relationships between nominal and measured exposure concentrations were used to interpolate exposure concentrations where samples were not collected (i.e. for three of the seven dilutions in vials). Differences between measured concentrations at the start and end of toxicity tests (Table 2) were expressed as the relative percentile difference (RPD) Eq. 1.

$$\text{RPD} = (\text{Difference}/\text{Average}) \times 100 \quad (1)$$

Results and Discussion

Linear relationships between initial nominal and measured final VCH concentrations were derived for groundwater and polynomial relationships were derived for 1,2-dichloroethane and chloroform test solutions. All had coefficients of determination (r^2) of >0.99; thus explaining more than 99% of the variation in measured concentrations. Loss of VCHs and inaccuracy during preparation of test solutions was evaluated by comparison of predicted (or nominal) concentrations and measured concentrations at the start of the toxicity testing. There was less than 50% difference between predicted and measured dilutions of contaminated groundwater in vials; however, for 1,2-dichloroethane and chloroform, differences between predicted and measured dilutions were between one and two orders of magnitude. In jars, differences between predicted and measured dilutions for contaminated groundwater, 1,2-dichloroethane and chloroform were all less than 50%.

Effects of the test solution preparation were larger for vials than jars, where considerably greater loss of VCHs was observed. The greater loss of VCHs for vials than for jars, could be explained by the greater number of dilutions

Table 2 Nominal and measured concentrations (in mg/L) of test chemicals and volatile chlorinated hydrocarbons (VCHs) in vials and jars

Treatment	Nominal concentration	Measured concentration		RPD
		Start	Final	
Vial	45	41.2	52.8	+24.8
Groundwater	10	9.22	10.2	+9.60
	2.5	2.12	2.37	+11.4
	0.5	0.45	0.68	+40.5
Vial	1,000	811	1,140	+33.7
1,2-dichloroethane	100	88.1	130	+38.4
	10	5.40	5.83	+7.70
	1	0.057	0.063	+10.0
Vial	300	177	209	+16.6
Chloroform	30	9.58	10.9	+12.9
	3	0.229	0.335	+37.6
	0.3	0.003	0.005	+50.0
Jar	45	51.3	44.1	−15.1
Groundwater	19.5	19.8	16.5	−18.2
	8.5	10.1	7.25	−32.9
	3.5	4.67	2.74	−52.1
Jar	1,000	516	368	−33.5
1,2-dichloroethane	300	150	158	+5.20
	100	69.6	51.2	−30.5
	30	18.5	13.2	−139
Jar	300	193	133	−36.8
Chloroform	100	49.1	27.1	−57.7
	30	29.4	11.9	−84.7
	10	10.7	6.42	−50.0

required, i.e. seven in vials compared to four in jars. Smaller solution volumes were also required for vials than jars. The observed differences between predicted and measured concentrations highlight the need to undertake analytical testing to confirm exposure concentrations of VCHs, rather than relying on nominal exposure concentrations.

Measured concentrations for each of the three test solutions in vials indicated that there was no loss of VCHs for the duration of toxicity testing, with slight increases reported, within the range of analytical variability (Table 2). Average analytical variability for blind duplicates for this analysis at this laboratory is typically 25%, when measured by RPDs. The results likely reflect analytical variability rather than reflecting a true, and somewhat improbable, increase in analyte concentrations. In jars, measured concentrations at the conclusion and start of testing indicated a loss of VCHs in all but one sample treatment (300 mg/L dilution of 1,2-dichloroethane). Concentrations were lower at the end of testing by, on average, 29%, 52% and 57% for groundwater, 1,2-dichloroethane and chloroform, respectively. The RPD metric is skewed when low concentrations are present. Although these results include a component of analytical variability (~25%), they also likely represent an actual decline in VCHs and exposure concentrations of test organisms from volatilisation. Jars were less effective than vials at maintaining constant exposure concentrations for test organisms and preventing loss of VCHs. The greater losses of VCHs from jars than vials were probably due to compounds escaping into headspace present in the jars, in accordance with the findings of Mayer et al. (2000). Conditions with zero headspace, as was the case with vials in the current study, would, as long as the seal was airtight, prevent partitioning of contaminants into the vapour phase and subsequent loss from the test solution. Jars used in the current study for toxicity testing contained approximately 50% headspace allowing partitioning of VCHs into the vapour phase and loss from test solutions. Concentrations of chloroform declined more than VCHs or 1,2-dichloroethane. This is consistent with the finding of Mayer et al. (2000), that the lower the boiling point, the greater the loss from solution, as the boiling point of chloroform is approximately 30% lower than for 1,2-dichloroethane.

The suitability of the two types of test vessels for use with the test organisms was evaluated by survival of

Table 3 Test acceptance criteria and survival of test organisms in artificial sea water (ASW) and filtered sea water (FSW) controls

Organism	Test acceptance criteria	ASW control	FSW control	Reference toxicant result
Alga	Cell yield ≥30,000 cells/mL	64,250 cells/mL	58,250 cells/mL	22.7 μg Cu^{2+}/L
Fish	≥80% survival in controls	80% survival	53% survival	Not applicable
Polychaete	≥90% survival in controls	100% survival	100% survival	Not applicable
Sea urchin	≥70% normal larvae in controls	91% normal	93% normal	9.1 μg Cu^{2+}/L
Oyster	≥70% normal larvae in controls	69% normal	83% normal	19.8 μg Cu^{2+}/L
Amphipod	≥90% survival in controls	86% survival	100% survival	3.53 mg NaDS/L

organisms in the two negative controls (i.e. artificial salt water – ASW and filtered salt water – FSW), in reference toxicants and maintenance of water quality parameters. In tests undertaken in both vials and jars, performance of reference toxicants were within the quality criteria (Table 3), dissolved oxygen content exceeded the minimum of 65% saturation and pH was maintained within the required range (7.5–8.3) throughout all tests (Table 1). Micro-algal population growth and sea urchin larval development tests, both undertaken in vials, met the test acceptance criteria (Table 3). However, percent normal development in the oyster ASW control was 69%, marginally below the test criterion of 70% (Table 3) in the ASW control, which may indicate that the artificial salt used in the test is only marginally suitable for the oyster. The oyster toxicity test was also extended from 48 to 72 h due to slow development of D-veliger shells, which often occurs in tests undertaken with this organism in winter, i.e. outside of the regular spawning season (Widdows 1993), as was the case in the current investigation. Overall, the 44 mL vials, sealed with Teflon^{TM}-lined lids and zero headspace, were suitable test vessels for small organism tests. In jars, survival rates exceeded control limits with the exception of the fish larval imbalance test in the FSW control and the amphipod survival test in the ASW control (Table 3). Amphipod survival was 86%, marginally below the control limit of 90%, as was the case for the oyster, the artificial salt used in the test may only be marginally suitable for the amphipod. As survival in the FSW control and two treatments for both chloroform and 1,2-dichloroethane was 100%, this lower survival could be an anomalous result and unrelated to the use of jars. Fish survival was below the control limit of 80% in the FSW control (53%) (Table 3). Each replicate contains only five organisms and is therefore, sensitive to loss of one organism resulting in a lower survival rate (80%). Fish survival in the lowest concentration treatments of 1,2-dichloroethane and chloroform were 80% and 87%, respectively, and therefore, met the control limits. Given this, it is argued that the results of the fish toxicity test are valid. Previous fish larval imbalance toxicity tests with *M. novemaculeata* used larvae greater than 60 days old (Cohen and Nugegoda 2000). In the present test, however, in order to meet the recommended maximum of 30 days duration for an early life stage test (USEPA 2002), the larvae were 27 days old. This difference in age may have influenced survival in the controls. This is a sensitive test on an early life stage which requires further development.

All toxicity tests will periodically fail to meet their acceptability criteria. Therefore, by conducting one set of trials, we cannot categorically state that the test vessel is not appropriate for a particular species, particularly as they only just fail to meet the acceptability criteria. The fact that control values are so close to meeting the acceptability criteria indicates that (1) repetition would show the acceptability criteria are met in most cases or (2) further modification may lead to the acceptance of the test vessel. Although previous studies have assessed the suitability of toxicity testing using algae in sealed vessels, the present study has shown that the use of sealed vessels for toxicity testing with VCHs is also suitable, based on the maintenance of suitable exposure conditions, for a diverse range of taxa including: urchins; bivalves; amphipods; and polychaetes, and potentially for fish larvae, for which further development of the test is required.

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