Australasian Bulletin of Ecotoxicology and Environmental Chemistry

The Official Bulletin of the Australasian Chapter of the Society of Environmental Toxicology and Chemistry – Asia Pacific

Volume 10

2025



AUSTRALASIAN BULLETIN OF ECOTOXICOLOGY & ENVIRONMENTAL CHEMISTRY

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RESEARCH PAPER

THE SOUTHWEST AUSTRALIAN TOXICITY TEST SUITE: DEVELOPMENT OF STANDARDISED CHRONIC TOXICITY TESTS FOR TEMPERATE FRESHWATER SYSTEMS IN A GLOBAL BIODIVERSITY HOTSPOT

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ABSTRACT

This paper presents a case study for the development of a novel toxicity test suite applicable to temperate freshwater systems in the Global Biodiversity Hotspot of Southwest Australia. The Southwest Australian Toxicity Test Suite comprises 8 standardised laboratory tests for the assessment of chronic toxicity, and was developed through the modification of pre-existing methods to suit Southwest Australian species and conditions. The suite comprises the following freshwater species tests: gastropod mollusc: *Glyptophysa georgiana,* cnidarian: *Hydra viridissima*, the cladocerans: *Simocephalus exspinosus* and *Ceriodaphnia* cf. *dubia*, and the four chlorophytes: *Tetradesmus obliquus, Chlamydomonas oviformis, Chlorella* sp. and *Scenedesmus* sp. All species are native to the Southwest Australian bioregion, with *G. georgiana* believed to be endemic.

The entire test-development cycle from field-collection of candidate species to the development and validation of laboratory chronic toxicity tests is presented, and standardised chronic toxicity test protocols and culturing methods are provided herein. It is intended that the Southwest Australian Toxicity Test Suite is used for the assessment of toxicity of contaminated discharges, leachates, spills and other anthropogenic inputs to freshwater receiving environments to inform environmental regulation and management decisions within this high ecological value bioregion. Toxicity datasets generated with the Southwest Australian Test Suite are also applicable for the derivation of single toxicant guideline values, thresholds and limits for the protection of aquatic ecosystems, using the contemporary species sensitivity distribution approach.

An assessment of comparative sensitivity of the individual test species to the reference toxicant copper (based on median effect concentrations – EC50s) from most sensitive to least sensitive was: *H. viridissima* > *T. obliquus* > *Scenedesmus* sp. > *C. oviformis* > *S. exspinosus* > *C.* cf. *dubia* > *Chlorella* sp. > *G. georgiana.*

Keywords: aquatic ecotoxicology, freshwater toxicants, local-species toxicity, site-specific, snail, hydra, cladoceran, microalgae.

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CONTENTS

Abstract 1
1. Introduction 4
2. Case study: Development of the Southwest Australian Toxicity Test Suite 7
2.1 Field-collection of candidate species 7
2.1.1 Identification of sites for candidate species collection 7
2.1.2 Field collection procedures 9
2.1.3 Sample transportation 12
2.2 Development of laboratory cultures with field-collected candidate species 12
2.2.1 Isolation of candidate species 13
2.2.2 Culture development and maintenance 13
2.2.3 Taxonomic identification of candidate species 13
2.2.4 Suitability of candidate species for inclusion in toxicity test method development phase 13
2.3 Toxicity test method development and test condition optimisation 15
2.3.1 Test acceptability criteria and corresponding test species tolerance ranges 15
2.3.2 Conclusion of method development phase 15
2.3.3 The Southwest Australian Toxicity Test Suite 17
3. Standardised toxicity test procedures for the Southwest Australian Test Suite 19
3.1 Test procedure: Gastropod mollusc, *Glyptophysa georgiana* (endemic to Southwest Australia) egg production impairment chronic toxicity test 22
3.1.1 Method summary 22
3.1.2 Organism culture 24
3.1.3 Equipment 24
3.1.4 Test preparation 24
3.1.5 Daily test procedure 26
3.1.6 Test acceptability criteria 28
3.1.7 Analysis of test data 29
3.2 Test procedure: Cnidarian, *Hydra viridissima* (Southwest Australian isolate) population growth inhibition chronic toxicity test 30
3.2.1 Method summary 30
3.2.2 Organism culture 32
3.2.3 Equipment 33
3.2.4 Test preparation 33
3.2.5 Daily test procedure 34
3.2.6 Test acceptability criteria 36
3.2.7 Analysis of test data 37
3.3 Test procedure: Cladoceran, *Simocephalus exspinosus* (Southwest Australian isolate) partial lifecycle chronic toxicity test 38
3.3.1 Method Summary 38
3.3.2 Organism culture 41
3.3.3 Equipment 41
3.3.4 Test preparation 42
3.3.5 Daily test procedure 42
3.3.6 Test acceptability criteria 45
3.3.7 Analysis of test data 46
3.4 Test procedure: Cladoceran, *Ceriodaphnia* cf. *dubia* (Southwest Australian isolate) partial lifecycle chronic toxicity test 47
3.4.1 Method Summary 49
3.4.2 Organism culture 49
3.4.3 Equipment 51

3.4.4 Test preparation 51
3.4.5 Daily test procedure 52
3.4.6 Test acceptability criteria 55
3.4.7 Analysis of test data 55
3.5 Test procedure: Microalgae, *Chlamydomonas oviformis*, *Tetradesmus obliquus*, *Scenedesmus* sp., and *Chlorella* sp. (Southwest Australian isolates) growth inhibition chronic toxicity tests 56
3.5.1 Method summary 57
3.5.2 Organism culture 57
3.5.3 Equipment 60
3.5.4 Test preparation 60
3.5.5 Daily test procedure 61
3.5.6 Test acceptability criteria 63
3.5.7 Analysis of test data 64
4. Comparative assessment of copper toxicity 65
Acknowledgements 65
References 66
Appendices 69
Appendix A.1: Culture development, maintenance and test condition optimisation for the gastropod mollusc test: *Glyptophysa georgiana* (Endemic to southwest Australia) 69
Appendix A.2: Standard operating procedure for culture maintenance for gastropod mollusc test: *Glyptophysa georgiana* (endemic to Southwest Australian) 71
Appendix A.3: Reference toxicant testing and establishment of a control chart for gastropod mollusc test: *Glyptophysa georgiana* (Endemic to southwest Australia) 72
Appendix B.1 Culture development, maintenance and test condition optimisation for Cnidarian test: *Hydra viridissima* (Southwest Australian isolate) 74
Appendix B.2: Standard operating procedure for culture maintenance for Cnidarian test: *Hydra viridissima* (Southwest Australian isolate) 76
Appendix B.3: Reference toxicant testing and establishment of a control chart for Cnidarian test: *Hydra viridissima* (Southwest Australian isolate) 79
Appendix C.1: Culture development, maintenance and test condition optimisation for Cladoceran test: *Simocephalus exspinosus* (Southwest Australian isolate) 81
Appendix C.2: Standard operating procedure for culture maintenance for Cladoceran test: *Simocephalus exspinosus* (Southwest Australian isolate) 84
Appendix C.3: Reference toxicant testing and establishment of a control chart for Cladoceran test: *Simocephalus exspinosus* (Southwest Australian isolate) 87
Appendix D.1: Culture development, maintenance and test condition optimisation for Cladoceran test: *Ceriodaphnia* cf. *dubia* (Southwest Australian isolate) 89
Appendix D.2: Standard operating procedure for culture maintenance for Cladoceran test: *Ceriodaphnia* cf. *dubia* (Southwest Australian isolate) 92
Appendix D.3: Reference toxicant testing and establishment of a control chart for Cladoceran test: *Ceriodaphnia* cf. *dubia* (Southwest Australian isolate) 95
Appendix E.1: Culture development, maintenance and test condition optimisation for four chlorophyte tests: *Tetradesmus obliquus*, *Chlamydomonas oviformis, Scenedesmus* sp. and *Chlorella* sp. (Southwest Australian isolates) 97
Appendix E.2: Standard operating procedure for culture maintenance for four chlorophyte tests: *Tetradesmus obliquus*, *Chlamydomonas oviformis*, *Scenedesmus* sp. and *Chlorella* sp. (Southwest Australian isolates) .. 100
Appendix E.3: Reference toxicant testing and establishment of control charts for four chlorophyte tests: *Tetradesmus obliquus*, *Chlamydomonas oviformis*, *Scenedesmus* sp. and *Chlorella* sp. (Southwest Australian isolates) 103
Appendix F: Preparation of GP-2 salts for test diluent and control water 106

1. INTRODUCTION

Southwest Australia is one of 36 Global Biodiversity Hotspots, defined as Earth's most biologically rich, yet threatened regions (CEPF 2022). The Southwest Hotspot, covering 356,700 km^2 (Figure 1), is Australia's only listed Hotspot, primarily identified for its exceptional floral variety (Myers et al. 2000). The level of endemism of riverine fauna is also amongst the highest on the planet with, for example, all of the native crayfish and bivalve molluscs and over 90% of fish that inhabit wetlands and waterways of the region found nowhere else in the world, and many of these species occur in very restricted ranges (DWER 2024a; Storer et al. 2020). River ecosystems of the region have evolved in isolation over millions of years to suit the characteristically long hot, dry seasons and relatively short periods of high rainfall. Most systems retreat to disconnected pools during the dry season, in many cases reliant on shallow groundwater and many species display specialised adaptations to these conditions.

The inland waters of the Southwest are subject to significant and increasing threat, as is the case for most freshwater systems around the world. A recent review of all species listed by the International Union for Conservation of Nature found that approximately one quarter of all freshwater species are threatened with extinction, including around 30% of freshwater crayfish, crabs and shrimps, and around 26% of freshwater fish (Sayer et al. 2025). In addition to the long-term pressures from clearing of native vegetation for development, water abstraction, introduced and invasive species, altered bushfire patterns and inputs of aquatic pollutants through urbanisation and industrialisation, the Southwest of Australia is facing unprecedented drying climate. This region has dried at one of the fastest rates on the planet (Masson-Delmotte et al. 2021). Since the 1970s, rainfall has declined by over 20%, average temperature has increased by around 1 °C, groundwater recharge has dropped by approximately 70% and flow in some streams has decreased by over 50% (DWER 2024b; SOTC 2024).

The risk from pollutants to Southwest aquatic ecosystems has increased significantly with the decline in runoff to waterways (from drying climate and water use), coupled with increased evaporation due to increasing temperature and reduced shade (cleared riparian vegetation). Reduced flows constrain the ability of rivers and creeks to dilute, disperse and ameliorate pollution, and for species to withstand impacts from toxicants in waste discharges, leachates and spills.

The Southwest region is resource-rich, with rapidly growing industries such as lithium exploration, mining and processing due to the high global demand for this technology-critical element (Batley and Campbell 2022). The environmental regulation and management of emissions to waterways from these operations in Australia follows the Water Quality Management Framework outlined in the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZG 2018) in line with the National Water Quality Management Strategy (Commonwealth of Australia 2018). A key component is the assessment of risk and impact to aquatic ecosystems, an approach also adopted by Canada (Environment and Climate Change Canada), Europe (European Commission – Environment), the USA (US Environmental Protection Agency) and the UK (UK Environment Agency), in which risks to aquatic environments are assessed and quantified through multiple lines of evidence.

In Australia, through the regulatory process, emissions to waterways are typically subject to a set of conditions aimed at maintaining or improving ecosystem health through specific criteria, such as toxicant threshold concentrations or guideline values. Compliance monitoring against the criteria is subsequently undertaken to ensure conditions are met and ecosystem health is not being impacted. Water quality criteria are often drawn from a national set of default guideline values for toxicants (for specific toxicants where guidelines exist). However, there are inherent risks to using default water quality guidelines to protect specific aquatic ecosystems (Trenfield et al. 2020; ANZG 2018; van Dam et al. 2014). Thus, the preferred approach to setting benchmarks or thresholds is through

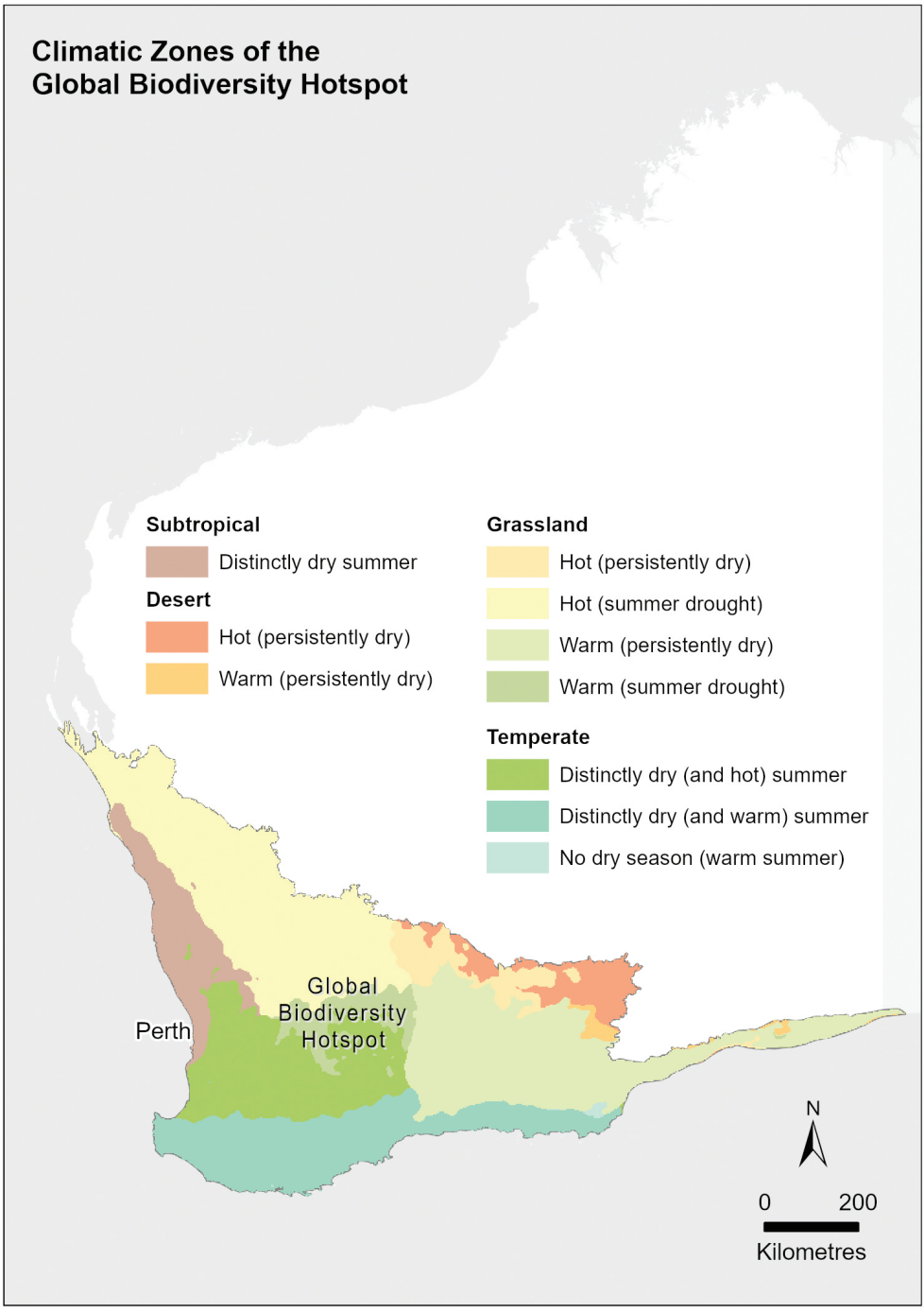


Figure 1: The Southwest Australia Global Biodiversity Hotspot and climate zones [data sources: Conservation International 2019; DCCEEW 2025; COA 2005]

the development of site or region-specific guideline values through toxicity testing using species, endpoints and conditions that are reflective of a specific site or region, provided the toxicity methods meet acceptable quality standards (ANZG 2018; Warne et al. 2025, 2018). Such an approach (local species, local water quality and consideration of local toxicity modifying factors), greatly increases confidence in the resulting guidelines (van Dam et al. 2019).

A review of current West Australian environmental regulatory processes and tools identified an absence of commercially available laboratory toxicity tests using species sourced from (and thus directly representative of) West Australian inland waterways. Given the uniqueness of the southwest bioregion, its high environmental value and rapidly expanding resource industry, priority was given to the development of a toxicity test suite that is environmentally representative (local species), has traditional endpoints for chronic effects, can be implemented under a range of conditions relevant to the waterways of the southwest bioregion and is available for use by all environmental practitioners (with methods publicly available) including environmental regulators, researchers and industry.

This paper presents the development of the Southwest Australian Toxicity Test Suite applicable to temperate freshwater systems in the southwest bioregion and Global Biodiversity Hotspot of Southwest Australia.

The test suite is suitable for:

- toxicity assessment of single chemicals for the derivation of site or region-specific water quality guideline values for the protection of aquatic ecosystems in the Southwest Australian bioregion
- toxicity assessment of complex mixtures (such as sewage discharge, mining wastewater, agricultural runoff, stormwater and industrial effluent), to determine dilution factors and/or treatment required prior to discharge to aquatic receiving environments in the Southwest Australian bioregion, in order to meet a designated level of protection
- use in direct toxicity assessment as an ecosystem receptor line of evidence in aquatic environmental impact and risk assessments.

Test development is presented in Section 2, individual standardised chronic toxicity test procedures are provided in Section 3, a comparative sensitivity assessment of test species to the reference toxicant, copper, is presented in Section 4 and standard operating procedures for culturing test species are provided in Appendices A to E.

2. CASE STUDY: DEVELOPMENT OF THE SOUTHWEST AUSTRALIAN TOXICITY TEST SUITE

The stages applied from field collection through the entire test development cycle are summarised in Figure 2 and described in the following sections.

2.1 FIELD-COLLECTION OF CANDIDATE SPECIES

2.1.1 Identification of sites for candidate species collection

Desktop analysis of a wide range of corporate and project datasets held by Department of Water and Environmental Regulation, including but not limited to geospatial (geological, meteorological, elevation, land use), ecological (aquatic fauna, terrestrial vegetation and in-stream habitat data) and hydrological and water quality datasets, was undertaken in 2020 to identify potentially suitable waterways for the collection of candidate species. Several river reaches within the Blackwood River, Donnelly River and Warren River catchments were short-listed for field assessment (Figure 3). Short-listing was based on the following criteria: a) minimal development in the reach and upstream catchment, b) good water quality, c) appropriate in-stream and riparian habitat, d) representativeness of the broader catchment, e) distribution of species, and f) practicability / feasibility for ongoing sample collection.

Subsequent in-field ground-truthing of numerous sites within the identified reaches of these catchments was undertaken during October 2020. Six sites were then selected for further screening of suitability for future candidate species collection. Screening incorporated an assessment of water quality for a range of parameters. *In situ* measurement of salinity, conductivity, dissolved oxygen and pH was undertaken using a multiparameter digital water quality meter (YSI-ProDSS). Unfiltered water samples were collected from each site for the assessment of alkalinity, water hardness (as mg/L $CaCO_3$), total metals/metalloids analysis (arsenic, cadmium, chromium, cobalt, copper, lead, lithium, manganese, mercury, molybdenum, nickel, selenium, zinc) in addition to arsenic and chromium speciation. Dissolved organic carbon (DOC) was measured in water samples (0.45 µm filtered) immediately after collection. Laboratory analysis was undertaken by the State Government Laboratory, Chem Centre, Western Australia using inductively coupled plasma atomic emission spectroscopy and mass spectrometry (ICP-AES and ICPMS) techniques. Parameters were screened against default guideline values for 99% and 95% level of species protection (ANZG 2018) (Table 1 and Table 2).

All metals and metalloid concentrations, alkalinity, DOC and hardness were less than the laboratory limits of reporting (LoR) in laboratory and field blanks (ultrapure water), indicating no contamination of the samples had occurred during sampling and analysis processes. Physico-chemical parameters were within typical ranges at all sites screened. However, samples collected from Saltwater Gully, Upper Donnelly and Chappel Bridge sites showed one or more metals were present at concentrations greater than the default guideline values for 99% species protection level (ANZG 2018). Trace levels of lithium were reported in water collected from all sites (0.0005 to 0.0018 mg/L). There are currently no default guideline levels for lithium. However, all levels were within reported natural concentration ranges for lithium in surface waters (0 to 0.04 mg/L – Aral and Vecchio-Sadus 2008) and the concentrations reported here are expected to be associated with the natural local geology. Based on the water quality screening, Yanmah Brook in the Donnelly River Catchment and East Brook, and Lefroy Brook (Redgum Bridge) sites in the Warren River catchment were considered suitable sites for subsequent candidate species and bulk water (for use as culture media) collection.

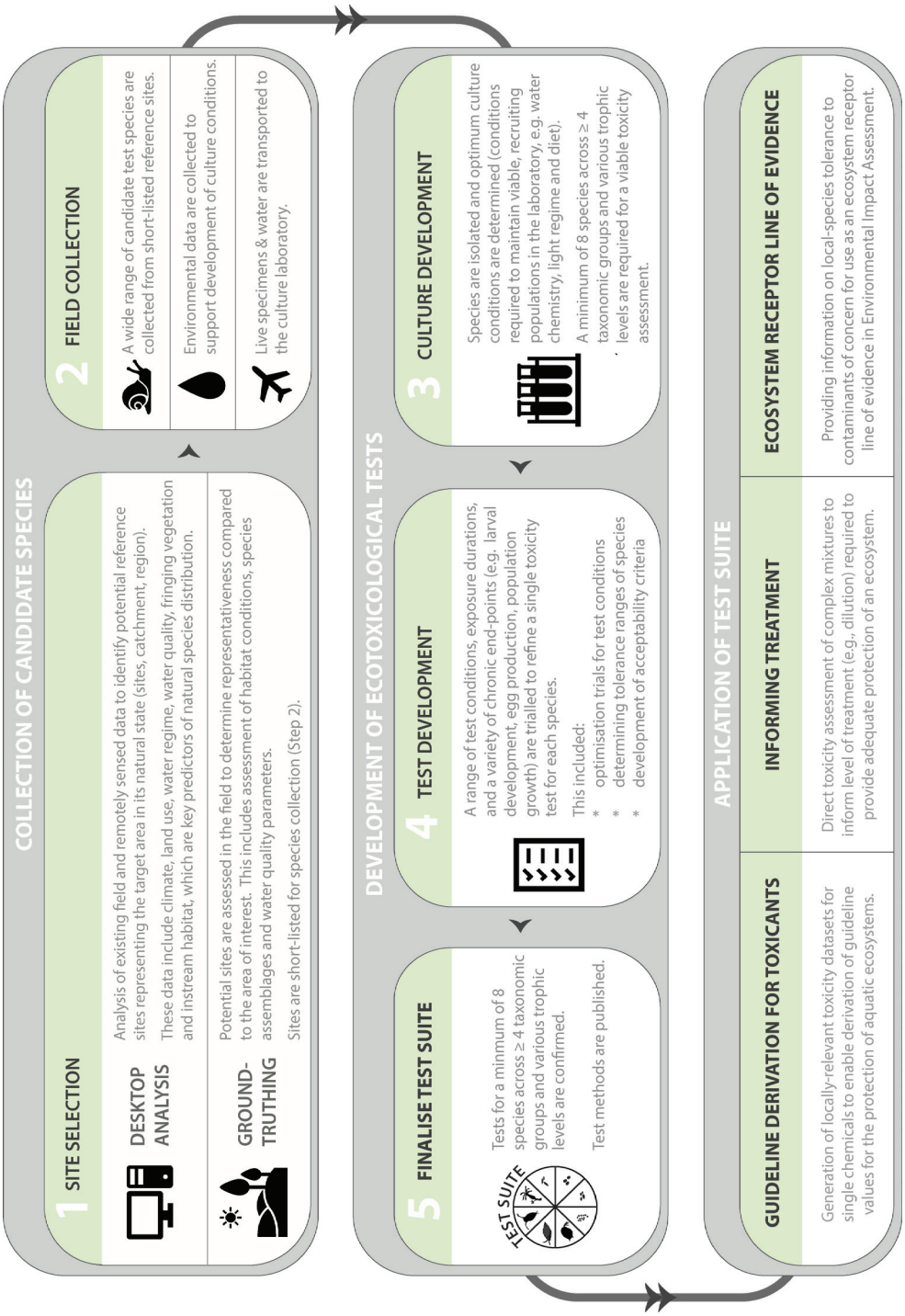


Figure 2: Summary of stages in the development of a suite of locally relevant toxicity tests for Southwest Australia – through collection of candidate species, test development and application

2.1.2 Field collection procedures

Methods for field collection of macroinvertebrate and algal species were chosen to capture as many different candidate species as possible. Macroinvertebrate samples (except hydra) were collected using 250 µm D-frame nets, with sweep and kick-netting as required through the array of different habitats occurring at each site. Samples were transferred to sorting trays for live picking by experienced field officers. Sampling followed standard AUSRIVAS methods within the South West Index of River Condition (Storer et al. 2020), although the size of the assessment area was varied to ensure adequate representation of all habitats (quantitative samples were not required for the purposes of the study). Predatory species were isolated from samples.

Samples of river water, macrophytes, charophytes, detritus and sediment were also collected to capture algae and any species that may have been missed in targeted macroinvertebrate collection, such as hydra which were found attached to macrophytes. Samples from each site were sorted into the following categories for transport: water samples containing organisms < 250 µm, water samples containing live-picked organisms > 250 µm, water samples containing macrophytes and charophytes, and clean sediment samples.

Candidate species sampling was undertaken three times during a four month period, with samples collected from Yanmah Brook (Donnelly River catchment) and East Brook (Warren River catchment) (Figure 3 and Figure 4). Bulk water samples (60 L) (0.45 µm filtered) to be used as test organism culture media were collected from Yanmah Brook (Donnelly River Catchment) at monthly intervals for 5 months (including a batch five days ahead of arrival of each batch of candidate species samples).

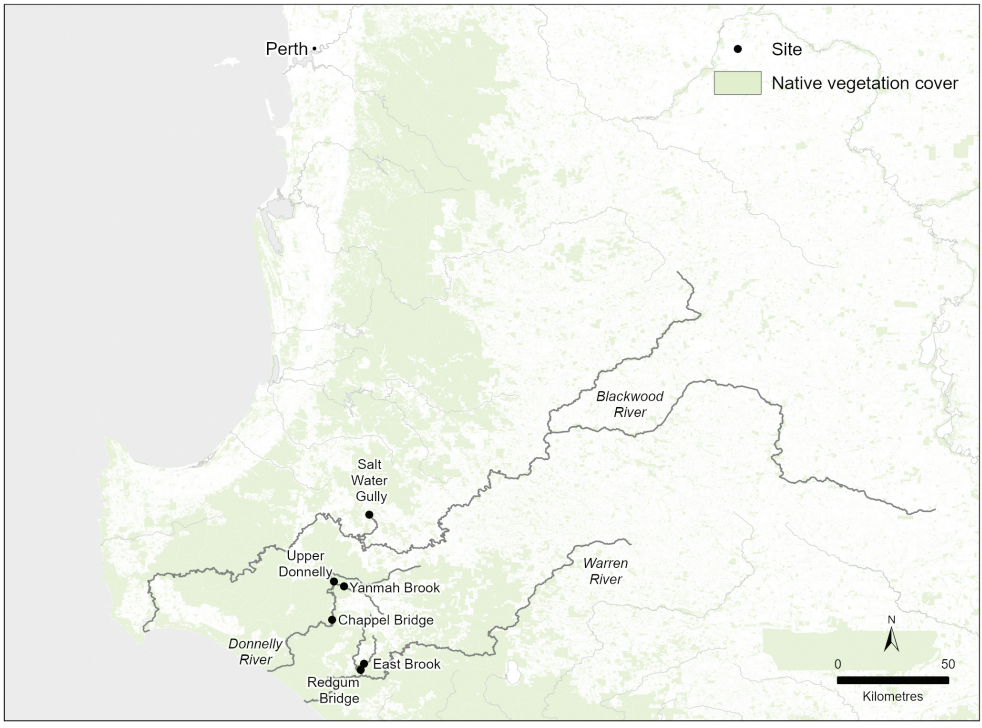


Figure 3: Water quality screening sites within the Blackwood, Donnelly and Warren River catchments (Data source – DWER 2024c; DCCEEW 2024)

Table 1: Water quality screening at six shortlisted sites within the Blackwood, Donnelly and Warren River catchments – physico-chemical water quality parameters.

Catchment	River	Site	Parameters							
			Salinity (psu)	Specific conductivity (mS/cm)	Dissolved Oxygen (% saturation)	Dissolved oxygen (mg/L)	pH	Alkalinity as $CaCO_3$ (mg/L)	Dissolved organic carbon (mg/L)	Hardness as $CaCO_3$ (mg/L)
Blackwood River	Saltwater Gully	Saltwater Gully (BR197HEST1)	0.15	0.322	86.6	8.83	6.9	33	8.0	41
Donnelly River	Manjimup Brook	Yanmah Brook (DR199MANJ)	0.26	0.535	92.8	9.78	7.39	41	8.0	73
	Donnelly River	Upper Donnelly (DR214DONN1)	0.4	0.818	81.8	8.69	6.98	33	15.0	110
	Donnelly River	Chapel Bridge (DR182DONN1)	0.22	0.462	96.4	10.34	7.04	17	6.9	62
Warren River	East Brook	East Brook (WR104EAST1)	0.28	0.584	98.3	10.25	7.39	43	5.6	74
	Lefroy Brook	Redgum Bridge (WR103LEFR1)	0.2	0.405	99.8	10.23	7.53	28	5.1	53
Field Blank			-	-	-	-	-	<1	<1.0	<1
Lab Blank			-	-	-	-	-	<1	<1.0	<1



Figure 4: Candidate species sampling sites: top: Yanmah Brook, bottom: East Brook

Table 2: Water quality screening at 6 shortlisted sites within the Blackwood, Donnelly and Warren River catchments – metals and metalloid analysis

		Parameters (mg/L)														
Catchment	**Site**	**Arsenic (III)**	**Arsenic (V)**	**Total cadmium**	**Total chromium**	**Chromium (VI)**	**Total cobalt**	**Total copper**	**Total lead**	**Total lithium**	**Total manganese**	**Total mercury**	**Total molybdenum**	**Total nickel**	**Total selenium**	**Total zinc**
Blackwood River	Saltwater Gully	<0.001	<0.001	0.0001	0.0005	<0.004	0.0014	0.0006	0.0002	0.0018	0.058	<0.00005	<0.0001	0.0008	<0.0002	<0.005
Donnelly River	Yanmah Brook	<0.001	<0.001	<0.0001	0.0002	<0.004	0.0003	0.0005	<0.0001	0.0005	0.011	<0.00005	<0.0001	0.0008	<0.0002	<0.005
	Upper Donnelly	<0.001	<0.001	<0.0001	0.0007	<0.004	0.0028	0.0008	<0.0001	0.001	0.024	<0.00005	<0.0001	0.0031	<0.0002	<0.005
	Chapel Bridge	<0.001	<0.001	<0.0001	0.0004	<0.004	0.016	0.0012	<0.0001	0.0018	0.12	<0.00005	<0.0001	0.01	<0.0002	0.012
Warren River	East Brook	<0.001	<0.001	<0.0001	0.0002	<0.004	0.0007	0.0004	<0.0001	0.0008	0.048	<0.00005	<0.0001	0.0008	<0.0002	<0.005
	Redgum Bridge	<0.001	<0.001	<0.0001	0.0002	<0.004	0.0002	0.0005	<0.0001	0.0008	0.024	<0.00005	<0.0001	0.0009	<0.0002	<0.005
Field Blank		<0.001	<0.001	<0.0001	<0.0001	<0.004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.00005	<0.0001	<0.0005	<0.0002	<0.005
Lab Blank		<0.001	<0.001	<0.0001	<0.0001	<0.004	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.00005	<0.0001	<0.0005	<0.0002	<0.005
99% SPL*		**0.001**	**0.0008**	**0.00006**		**0.00001**	**0.0014**	**0.001**	**0.001**		**1.2**	**0.00006**	**0.034**	**0.008**	**0.005**	**0.0024**
95% SPL*		**0.024**	**0.013**	**0.0002**		**0.001**		**0.0014**	**0.0034**		**1.9**	**0.0006**		**0.011**	**0.011**	**0.008**

** Default guideline values for 99% and 95% level of species protection (ANZG 2018). Note: total arsenic was also measured and data from all sites was below LoR (0.0001 mg/L)*

In situ measurement of salinity, conductivity, dissolved oxygen and pH was undertaken at each sample collection site immediately prior to sampling for candidate species and bulk water samples using a multiparameter digital water quality meter (YSI-ProDSS) (data can be supplied on request).

2.1.3 Sample transportation

Field-collected samples containing candidate species were express-couriered by air to Ecotox Services Australia laboratory NSW, Australia. Snails were transported in 1 L sealed buckets containing 500 mL water, hand-picked invertebrate specimens were transported in family groups in 4 L polyethylene bags (food-grade) with approximately 60% airspace and samples of macrophytes, sediment and detritus were transported in 10 L polyethylene bags with approximately 30% airspace (Figure 5). A small proportion of 100% oxygen (fish-grade) was added to bags to increase longevity for transport, but without risking hyperoxia.

All samples were double bagged and placed on foam mats over ice bricks in cool boxes for transportation by air courier. Bulk water was transported in 3 x 20 L aged plastic containers on ice bricks in cool boxes by surface courier.



Figure 5: Assortment of containers for transport of specimens to the laboratory

2.2 DEVELOPMENT OF LABORATORY CULTURES WITH FIELD-COLLECTED CANDIDATE SPECIES

Immediately upon arrival at the laboratory, field-collected samples were measured for physico-chemical parameters (Table 3) and left to acclimatise overnight prior to sorting for candidate species.

Fresh field-collected bulk water to be used as the initial culture media (60 L) (0.45 µm filtered) from Yanmah Brook (Donnelly River Catchment) was provided to the laboratory at monthly intervals for five months (including a batch five days ahead of arrival of candidate species samples) and refrigerated until required.

Table 3: Physico-chemical parameters of water from field-collection sites

Parameter	Field-collection site	
	Yanmah Brook	East Brook
pH	7.4	7.4
Electrical conductivity (µS/cm)	535	584
Dissolved organic carbon (mg/L)	8.0	5.6
Hardness as $CaCO_3$ (mg/L)	73	74

2.2.1 Isolation of candidate species

Field-collected samples containing water, various macrophyte and charophyte species, detritus and sediment collected from Yanmah Brook (Donnelly River Catchment) and East Brook (Warren River Catchment) were examined in trays using a stereo dissecting microscope and candidate invertebrate species were isolated into separate aquaria containing acclimatised bulk water from Yanmah Brook. Microalgal species were isolated from bulk water samples collected from Yanmah and East brooks using a compound microscope with the aim of establishing as many axenic single-species cultures as possible. The isolation procedure for microalgal species followed multiple stages over a ten month period comprising numerous weekly inoculations of mixed cultures to maintain microalgae in exponential growth phase (when cultures are visibly green in colour), with concurrent repeated plating out of mixed-species cultures onto agar plates, followed by separation of individual colonies. Single species colonies were confirmed by microscopic examination. Detailed procedures for the isolation of test organisms are provided in Appendix A.1 to E.1.

2.2.2 Culture development and maintenance

Feeding trials were initiated immediately for each invertebrate candidate species in parallel to a series of trials to determine if cultures of each species could be maintained in laboratory-produced water. Subsequently, a series of additional culture development and maintenance trials were undertaken for each candidate species over a period of several months.

Once single-species colonies of microalgae candidate species were achieved, liquid single-species cultures were prepared using inoculum techniques in sterile USEPA media (Appendix E.2). Culture development and maintenance trial results for all species are provided in Appendices A.1 to E.1.

2.2.3 Taxonomic identification of candidate species

Identification of invertebrate candidate species was undertaken in the laboratory and confirmed by taxonomic experts. Mollusc identities were confirmed by Dr. S. Clarke (Invertebrate Identification, NSW). Cladoceran identities (*Simocephalus exspinosus, Ceriodaphnia laticaudata and Ceriodaphnia* cf. *dubia* were determined by Dr. R. Shiel (University of Adelaide Environment Institute, SA) and the identity of *S. exspinosus* was confirmed by Dr A. Kotov (Moscow State University Institute of Ecology and Evolution, Russia). Preliminary taxonomic identification of candidate algal species based on morphology was provided by M. Przybylska (Algae Test Consulting, Victoria). Algal identification was clarified by Dr M. Larsson and D. Halliday (Department of Water and Environmental Regulation, WA) using morphological and phylogenetic techniques. Single-species cultures were confirmed.

2.2.4 Suitability of candidate species for inclusion in toxicity test method development phase

Eleven candidate species isolated within the laboratory were assessed for suitability for inclusion in toxicity test method development phase (Section 2.3). Eight species were shortlisted for the development of standardised toxicity tests. The rationale for selection of a species for the method development phase is provided (Table 4).

Table 4: Suitability matrix for inclusion of candidate species to test method development phase

Phyla	Candidate species	Standardised methodology available for adaptation with candidate species	Comment on suitability for toxicity test development	Selected for SW Australian Test Suite
Mollusca	Freshwater limpet: *Ancylastrum cumingianus*	Not known	Unsuitable Cultures unsuccessful due to inability to feed in the laboratory.	no
	Pulmonate snail: *Glyptophysa georgiana*	Trenfield et al. (2020); Hogan et al. (2010); USEPA 2002a	Suitable Culture trials (Appendix A.1) were successful	yes
Cnidaria	Green hydra: *Hydra viridissima*	Trenfield et al. (2020); Reithmuller et al. (2003)	Suitable Culture trials (Appendix B.1) were successful	yes
Arthropoda	Cladoceran: *Chydorus* sp.	USEPA (2002b); Bailey et al. (2000)	Unsuitable Initial culture trials were successful, but this species was considered unsuitable as a test organism due to low fecundity.	no
	Cladoceran: *Simocephalus exspinosus*		Suitable Culture trials (Appendix C.1) were successful	yes
	Cladoceran: *Ceriodaphnia* cf. *dubia*		Suitable Culture trials (Appendix D.1) were successful	yes
	Cladoceran: *Ceriodaphnia laticaudata*		Suitable – not used Considered suitable for first 4 months of culture development until an epibiont hydrozoan infestation of the carapace occurred resulting in low fecundity and poor motility.	no
Chlorophyta	Microalga: *Tetradesmus obliquus*	Trenfield et al. (2020); OECD (2006); USEPA (2002a,b); Stauber (1995)	Suitable Culture trials were successful (Appendix E.1)	yes
	Microalga: *Chlamydomonas oviformis*			yes
	Microalga: *Scenedesmus* sp.			yes
	Microalga: *Chlorella* sp.			yes

2.3 TOXICITY TEST METHOD DEVELOPMENT AND TEST CONDITION OPTIMISATION

A series of test condition optimisation trials (Table 5) were undertaken on the shortlisted eight species in relation to test conditions and endpoints associated with pre-existing test methodology. Detailed trial results for each species are presented in appendices A.1 to E.1.

2.3.1 Test acceptability criteria and corresponding test species tolerance ranges

During test condition optimisation trials (Appendices A.1 to E.1), test acceptability criteria (modified from existing test methodologies) were developed for the shortlisted species and species tolerance ranges were determined for the physico-chemical parameters shown in Table 6. The defined test acceptability criteria (column 2) are met in the controls when test conditions fall within the defined physico-chemical tolerance ranges shown (column 3). The resulting standardised protocols (Section 3) are applicable for, and can be modified to suit local conditions within the tolerances specified.

2.3.2 Conclusion of method development phase

A toxicity test suite was developed comprising eight standardised toxicity tests (Figure 6). The tests were developed with traditional endpoints for chronic effects, through the modification of pre-existing protocols. The test suite represents a relatively diverse group of animals and plants, comprising four invertebrate species (*Glyptophysa georgiana, Ceriodaphnia* cf *dubia, Simocephalus exspinosus and Hydra viridissima*) spanning three orders (Hygrophila, Diplostraca and Anthoathecata) and three phyla (Mollusca, Cnidaria and Arthropoda); and four microalgal species (*Chlamydomonas oviformis,*

Table 5: Test condition optimisation trials undertaken

	Test species							
	Pulmonate snail *G. georgiana*	Green hydra *H. viridissima*	Cladoceran *S. exspinosus*	Cladoceran *C.* cf. *dubia*	Microalga *T. obliquus*	Microalga *C. oviformis*	Microalga *Scenedesmus* sp.	Microalga *Chlorella* sp.
	Endpoints investigated							
Test conditions	Egg production	Specific growth rate	Time to first brood and # neonates produced	Time to first brood and # neonates produced	Cell yield	Cell yield	Cell yield	Cell yield
Test media / diluent type	✓	✓	✓	✓	*	*	*	*
Feeding regime	✓	✓	✓	✓	*	*	*	*
Test duration	✓	✓	✓	✓	*	*	*	*
Test media temperature	✓	✓	✓	✓	✓	✓	✓	✓
Test media electrical conductivity	✓	✓	✓	✓	✓	✓	✓	✓
Test media hardness (mg/L $CaCO_3$)		✓	✓	✓				
Reference toxicant exposure (copper sulphate)	✓	✓	✓	✓	✓	✓	✓	✓

** test conditions already well established (USEPA 2002b)*

Table 6: Test acceptability criteria and corresponding species tolerance ranges

Test species	Test Acceptability Criteria (controls)	Physico-chemical tolerance ranges in test media/diluent	Physico-chemical criteria selected for test media/ diluent and control water for the standardised Southwest Australian test suite***
Pulmonate snail: *G. georgiana** **[96 h test]**	≥ 80% non-immobilised snails Mean number of egg-masses ≥ 3 per live snail Mean number of eggs per egg-mass ≥ 5 96 h EC50 estimate for the copper reference toxicant must fall within lower and upper warning limits determined and presented in Appendix A.2	Temperature: 24-26 °C ** Electrical conductivity: 210-3000 µS/cm	Temperature: 25 ± 1 °C Electrical conductivity: 500 ± 50 µS/cm pH: 7.6-8.2 Dissolved oxygen concentration: 90-105% saturation (7.4-8.6 mg/L)
Green hydra: *H. viridissima* **[96 h test]**	Mean number of normal hydra ≥ 20 (population growth rate of 0.17 hydra/day) 96 h EC50 estimate for the copper reference toxicant must fall within lower and upper warning limits determined and presented in appendix B.2	Temperature: 23-28 °C Electrical conductivity: 120-5000 µS/cm Hardness: 80-180 mg/L $CaCO_3$	Temperature: 25 ± 1 °C Electrical conductivity: 400 ± 40 µS/cm pH: 7.6-8.2 Dissolved oxygen concentration: 90-105% saturation (7.4-8.6 mg/L)
Cladoceran: *S. exspinosus* **[10 day test]**	≥ 80% non-immobilised cladocerans Mean number of young per non-immobilised female ≥ 15 60% of non-immobilised control organisms must produce ≥ 3 broods 10 day IC50 estimate for the copper reference toxicant must fall within lower and upper warning limits determined and presented in appendix C.2	Temperature: 24-26 °C Electrical conductivity: 80-2000 µS/cm Hardness: 80-180 mg/L $CaCO_3$	Temperature: 25 ± 1 °C Electrical conductivity: 180 ± 20 µS/cm pH: 7.8-8.3 Dissolved oxygen concentration: 90-105% saturation (7.4-8.6 mg/L)
Cladoceran: *C.* cf. *dubia* **[10 day test]**	≥ 80% non-immobilised cladocerans Mean number of young per non-immobilised female ≥ 15 60% of non-immobilised control organisms must produce ≥ 3 broods 10 day IC50 estimate for the copper reference toxicant must fall within lower and upper warning limits determined and presented in appendix C.2	Temperature range: 24-26 °C Electrical conductivity: 80-3500 µS/cm Hardness: 80-180 mg/L $CaCO_3$	Temperature: 25 ± 1 °C Electrical conductivity: 180 ± 20 µS/cm pH: 7.8-8.3 Dissolved oxygen concentration: 90-105% saturation (7.4-8.6 mg/L)

Table 6 continued: Test acceptability criteria and corresponding species tolerance ranges

Test species	Test Acceptability Criteria (controls)	Physico-chemical tolerance ranges in test media/diluent	Physico-chemical criteria selected for test media/ diluent and control water for the standardised Southwest Australian test suite***
Microalgae: *T. obliquus, C. oviformis, Scenedesmus* sp., *Chlorella* sp. **[72 h test]**	Mean algal cell density in the controls > 160,000 cells/mL The % coefficient of variation ≤ 20% 72 h IC50 estimate for the copper reference toxicant must fall within lower and upper warning limits determined and presented in Appendix E.3	Temperature range: 21-28 °C Electrical conductivity: 100-3000 µS/cm	Temperature: 25 ± 1 °C pH: 7.5 ± 0.1

** There was no difference in egg production and physico-chemical tolerance range for the test durations investigated (96 h and 7 days). As such, 96 h test duration will be applied for the resulting toxicity test. **range may be broader – snail trials were limited due to low numbers of snails available within the timeframe of the project. It is recommended that a testing facility adopting the pulmonate snail method allows up to one year to build up sufficient numbers. ***these criteria can be modified to suit local conditions (within the tolerance ranges specified)*

Tetradesmus obliquus, Scenedesmus sp. *and Chlorella* sp.) spanning three orders (Chlamydomonadales, Sphaeropleales and Chlorellales) within the phylum Chlorophyta (green algae). The greater the taxonomic diversity within the test suite, the greater the potential to represent a range in physiologies and natural sensitivities of biota from the region. Therefore, taxonomic diversity represented in the Southwest Australian Toxicity Test Suite was maximised, within the limitations of species that: a) were relevant to the region, b) wfere able to be laboratory cultured, and c) for which standardised methodologies existed (with traditional endpoints for chronic effects) that could be modified and further developed with Southwest Australian species and local conditions.

While test organisms were ultimately sourced from waterways in the Warren and Donnelly River systems in southwest Australia, their broader environmental relevance to the southwest region was confirmed through analysis of data on the distribution of species and habitats, including DWER biological and water information datasets (DWER 2024a,b), AlgaeBase (Guiry and Guiry 2022), Atlas of Living Australia (ALA 2022), Catalogue of Life (Bánki et al. 2024), Integrated Taxonomic Information System (ITIS 2022) and the Global Biodiversity Information Facility (GBIF 2022).

Through the test suite development phase, species tolerances to a range of physico-chemical parameters were determined, such that the individual tests can be modified to suit a range of conditions represented in other regions of Australia.

The resulting ecotoxicity test procedures for the eight species are presented in Section 3 with sensitivity assessment being undertaken with the widely used reference toxicant, copper (Section 4). It is intended that each of the individual ecotoxicity test procedures outlined in Section 3 serves as a standalone protocol which can be used within an appropriate ecotoxicity testing facility.

2.3.3 The Southwest Australian Toxicity Test Suite

The Southwest Australian Toxicity Test Suite is presented in Figure 6. This graphic also illustrates the taxonomy and niche occupation (habitat and feeding strategies) of the eight individual test species to indicate environmental relevance.

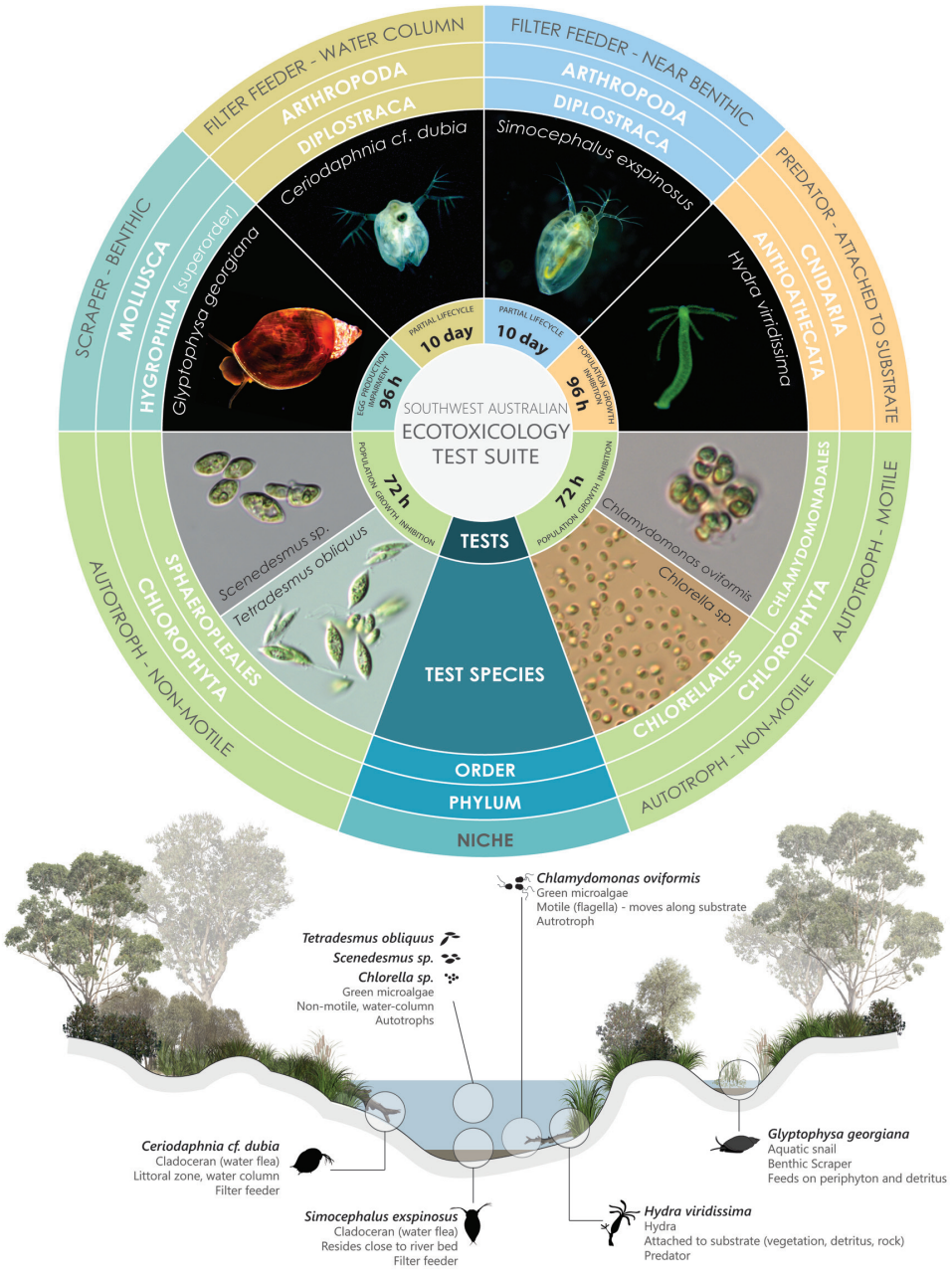


Figure 6: The Southwest Australian Toxicity Test Suite

3. STANDARDISED TOXICITY TEST PROCEDURES FOR THE SOUTHWEST AUSTRALIAN TEST SUITE

The following standardised toxicity test procedures are suitable for estimation of the chronic toxicity of single chemicals; e.g. for the derivation of environmental thresholds and criteria, in addition to the assessment of complex mixtures such as effluents, leachates, receiving surface waters, groundwaters and elutriates of sediments. The procedures can be applied as a screening test to undiluted test material or as a dilution series test to examine the degree of toxicity exhibited.

If maximum sample holding time restrictions do not apply (e.g. for single chemical testing), a range finding test may first be undertaken to determine the most appropriate test concentrations for a definitive test (USEPA 2002a). If sample holding time restrictions apply (e.g. for effluents), a broad range of test concentrations should be tested wi2thout a prior range-finding test. For a definitive full dilution series test, a dilution factor of 0.5 is commonly applied e.g. 100%, 50%, 25%, 12.5%, 6.3% 3.2%, 1.6% test concentrations. Additional test concentrations should be incorporated to address specific criteria such as the concentration of an effluent at the edge of a mixing zone. The number of test concentrations should also be modified to meet the requirements of statistical procedures for environmental guideline derivation methodology for the protection of aquatic ecosystems.

A summary of toxicity test conditions and criteria for the eight individual tests is presented in Table 7. Detailed standardised test procedures for each species are provided in sections 3.1 to 3.5.

Table 7: The test suite: toxicity test conditions and criteria for eight species

	Test organisms							
	Southwest Australian endemic	Southwest Australian isolates						
	Pulmonate snail	Green hydra	Cladocerans		Green algae			
Test conditions and criteria	*Glyptophysa georgiana*	*Hydra viridissima*	*Simocephalus exspinosus*	*Ceriodaphnia* cf. *dubia*	*Chlamydomonas oviformis*	*Tetradesmus obliquus*	*Scenedesmus* sp.	*Chlorella* sp.
Origin of test organism	East Brook, southwest Australia 2020-2021		East Brook & Yanmah Brook southwest Australia 2020-2021					
Test description	Egg-mass production impairment	Population growth inhibition	Partial lifecycle test		Algal growth inhibition			
Test endpoint(s)	# of egg-masses and eggs/mass	Population growth rate	Immobilisation and reproduction		Cell yield (determined via cell counts or absorbance at 750 nm)			
Test organism life-stage/age	Mature adult > 8 mm	Hydroid with single bud with tentacles	< 24 h		Inoculum prepared from 4-7 day old stock cultures			
Test duration	96 h		Until 60 % of non-immobilised control organisms have 3 broods (max 10 days)		72 h			
Test regime	Static, 24 h renewal				Static non-renewal			
Temperature	25 ± 1 °C							
Light quality and intensity	Cool white, fluorescent lighting (800 ± 160 Lux)		Cool white LED lighting (800 ± 160 Lux)		Cool white LED lighting (4440 – 8800 Lux)			
Photoperiod	16 h light: 8 h dark				12 h light: 12 h dark			
Chamber size	600 mL	50 mL	100 mL	20 mL	20 mL			
Test solution volume	500 mL	30 mL	80 mL	18 mL	10 mL			
# organisms per replicate	2	10	1		10,000 cells/mL			
# replicates per treatment	4	4	10		4 (8 replicates for primary control)			
Feeding regime	1 flake Sera Vipan per live snail daily	Fed brine shrimp nauplii daily	*Raphidocelis and* freeze dried *Dunaliella*, fed daily with each solution renewal		n/a			
Test chamber aeration	Gentle bubbling (60-100 bubbles/min)	No aeration	No aeration		No aeration. Vials shaken twice daily			
Diluent	20% mineral water: 80% deionised water with vitamin B12 and selenium supplements				USEPA Media (USEPA 2002b)			

Table 7 continued: The test suite: toxicity test conditions and criteria for 8 species

	Test organisms			
	Southwest Australian endemic	Southwest Australian isolates		
	Pulmonate snail	Green hydra	Cladocerans	Green algae
Test concentrations	Minimum of 6 test concentrations plus control			
Reference toxicant	31, 63, 125, 250 µg Cu/L 2 reps / treatment	1.1, 3.3, 10, 30 µg Cu/L 2 reps / treatment	6.3, 12.5, 25, 50, 100 µg Cu/L 5 reps / treatment	6.3, 12.5, 25, 50 µg Cu/L for *Scenedesmus* sp., *T. obliquus*, *Chlorella* sp. 6.3, 12.5, 25, 50, 100 µg Cu/L for *C. oviformis*. 3 reps / treatment
Test acceptability criteria	i) ≥ 87.5 % non-immobilised control organisms ii) Mean # eggs masses per live snail in controls ≥ 3 iii) Mean # eggs per egg-mass > 5	i) Mean # normally developed hydroids in the controls ≥ 20 (growth rate of 0.17 hydra/day)	i) ≥ 80 % non-immobilised control organisms ii) Mean # offspring per non-immobilised female in controls ≥ 15 iii) 60 % of non-immobilised control organisms must produce ≥ 3 broods	i) Mean cell density of ≥ 160,000 cells per mL in controls ii) Variability (CV %) ≤ 20 % in controls
Test modified from	Trenfield et al. (2020); Hogan et al. (2010); USEPA (2002a).	Trenfield et al. (2020); Riethmuller et al. (2003); Hyne et al. (1996).	USEPA (2002b); Bailey et al. (2000).	Trenfield et al. (2020); USEPA (2002 a,b); OECD (2006); Stauber (1995).

3.1 TEST PROCEDURE: GASTROPOD MOLLUSC, *GLYPTOPHYSA GEORGIANA* (ENDEMIC TO SOUTHWEST AUSTRALIA) EGG PRODUCTION IMPAIRMENT CHRONIC TOXICITY TEST

The test species, *Glyptophysa georgiana* (Quoy and Gaimard, 1832) (Figure 7) is a freshwater pulmonate gastropod snail limited in distribution to southwest Western Australia (Ponder et al. 2022). *G. georgiana* inhabits billabongs, swamps, rivers and creeks with low flow, residing on vegetation and woody debris and feeding on algae and detritus. Adults are 8 to 12 mm from tip of shell apex to spire, laying round to kidney shaped gel egg-masses, containing up to 17 eggs per mass. Within the laboratory, eggs were observed to hatch 4 to 5 days post-laying, with most to all eggs successfully hatching. Maturity is reached at 12 to 16 weeks post-hatch.

3.1.1 Method summary

This standardised test procedure can be used to estimate the chronic toxicity of single chemicals, complex effluents, leachates, receiving waters and elutriates of sediments to the freshwater pulmonate snail, *G. georgiana* (WA endemic). The procedure is based on the methodology described in Trenfield et al. (2020) and Hogan et al. (2010) for the Northern Territory pulmonate *Amerianna cumingi* and has been adapted for use with *G. georgiana* and temperate southwest conditions (this study). *G. georgiana* is notably smaller and produces fewer eggs per egg mass than *A. cumingi*. Adaptations to test methodology include modified test acceptability criteria to reflect these differences, in addition to various test conditions such as test solution volume, test temperature and feeding regime. In this test, mature egg-laying snails are exposed to the test material at a range of dilutions, and effects on egg mass production and number of eggs per mass are assessed (Figure 8).

Test design comprises four replicates per treatment, each with two randomly selected mature snails 8 to 12 mm (length). Test animal condition, number of egg-masses, and number of eggs per mass are assessed every 24 hours until the test is terminated at 96 hours.

This procedure describes routine testing. Test conditions and acceptability criteria are provided in Table 8. Modifications may be made to test requirements to suit region or site-specific conditions within the tolerance ranges presented in Table 6 (Section 2.3.1).

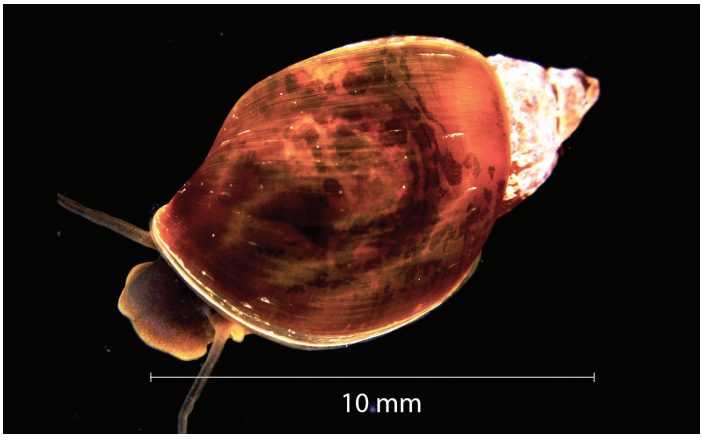


Figure 7: *Glyptophysa georgiana* (adult).
Photo credit: D. Halliday

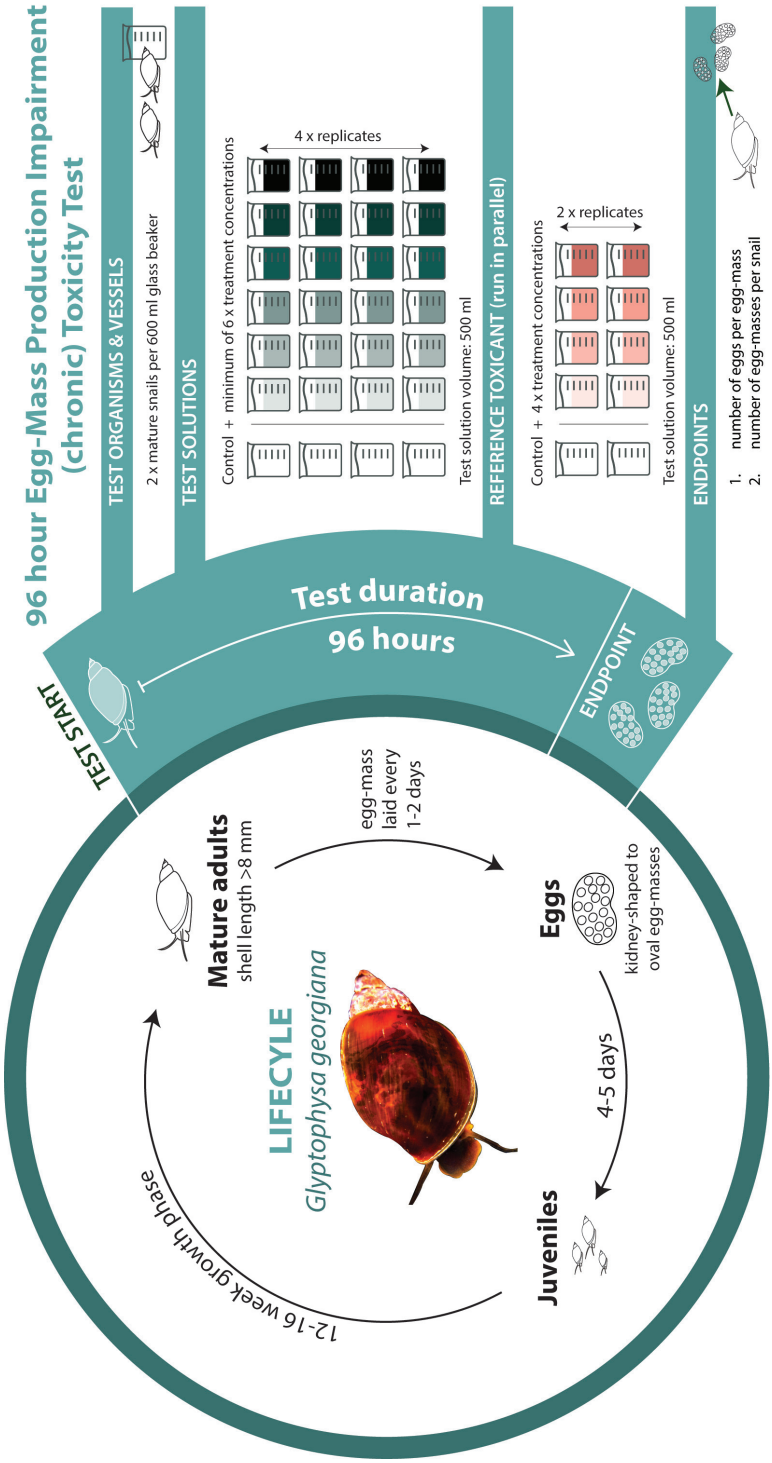


Figure 8: ***Glyptophysa georgiana*** **lifecycle and toxicity test experimental design details**

3.1.2 Organism culture

Snails (*G. georgiana)* used for ecotoxicity testing are maintained in 30 L (minimum volume) glass aquaria with a flow-through filtration system containing pre-conditioned mains water at a temperature of 25 ± 1 ºC, pH range of 7.6 to 8.2, dissolved oxygen of 90 to 105% saturation (7.4 – 8.6 mg/L) and a conductivity of 250 ± 50 µS/cm. Snail cultures should be in good health to ensure an adequate supply of mature egg-laying snails for testing and thus, the quality of the tests performed. The primary indicator of snail health is egg-mass production. Healthy snails will produce an egg-mass containing up to 17 eggs at least every second day, eggs will hatch 3 to 5 days from mass being deposited, with most or all eggs hatching. Detailed methodology on culture development is provided in Appendix A.1 and a Standard operating procedure for culture maintenance: Gastropod mollusc, *Glyptophysa georgiana* is provided in Appendix A.2. Source cultures of *G. georgiana* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Drive, Lane Cove, NSW, Australia, 2066; info@ecotox.com.au.

3.1.3 Equipment

- constant temperature chambers with light cycling
- pre-prepared diluent (dilute mineral water, DMW)
- pH, temperature, conductivity and dissolved oxygen meters (and associated calibration fluids)
- borosilicate glassware including 100, 250 and 600 mL beakers, 1 L measuring cylinders, 1, 2 and 5 L glass screw-cap bottles
- 50 mL Petri dishes
- forceps
- Sera Vipan Tropical Flakes (or equivalent, without dyes or preservatives)
- autopipettors (range: 10 to 5000 µL) with polyethylene tips.
- plastic trays
- stereo dissecting microscope

3.1.4 Test preparation

Test diluent and control water

Test diluent and control water is a standard dilute mineral water (DMW): 20% mineral water: 80% deionised water (USEPA 2002a), prepared by mixing 3.2 L deionised water with 800 mL mineral water in a 5 L bottle at least 4 hours prior to test commencement, to ensure dissolved carbon dioxide and pH have reached equilibrium. This is then modified with the addition of GP-2 salts (USEPA 2002b, Appendix F) while stirring with a magnetic stirrer to raise the electrical conductivity to 500 ± 50 µS/cm. Modified DMW is to be used within 4 days.

Note: where a toxicity assessment requires a field-collected diluent, e.g. upstream of a discharge point, the field-sourced diluent may not be optimal with respect to the health of the test organisms, resulting in test acceptability criteria (Table 8) not being met. As such, test design should include negative control treatments using both modified DMW and field-sourced diluents that can be statistically compared.

Sample preparation: effluents, receiving surface waters, groundwaters and leachates

Two litres of test material (sample) is required per treatment. This may need to be pre-filtered through a 60 µm mesh (stainless steel for samples where organic contaminants may be present and nylon where

Table 8: Summary of test conditions and test acceptability criteria for the snail, *G. georgiana* egg-mass production impairment (chronic) test

Test parameter	Test condition
Test species	*Glyptophysa georgiana* (Southwest Australian isolate)
Test type	Static renewal
Test duration	96 h
Temperature	25 ± 1 °C
Light quality	Cool white, fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16 h light: 8 h dark
Test chamber size	600 mL
Test solution volume	500 mL
Renewal of Test Solutions	Static non-renewal, or renewal every 24 h
Age of test organisms	Mature adult, 8-12 mm
No. of organisms per replicate	2
No. of replicates per treatment	4
No. of organisms per treatment	8
Feeding regime	Fed 1 flake Sera Vipan per live snail, daily
Test chamber cleaning	Rinsed daily, if required
Test chamber aeration	Gentle bubbling (60-100 bubbles/min) via Pasteur pipette
Dilution water	Dilute Mineral Water (DMW), prepared by mixing 20% Perrier mineral water with deionised water, with conductivity raised to 500 ± 50 µS/cm
Test concentrations	Minimum of 6 concentrations plus a control
Dilution series	≥ 0.5 dilution series (e.g. 100, 50, 25, 12.5 and 6.25%)
Endpoint	Egg-mass and eggs per mass
Reference Toxicant	$CuSO_4.5H_2O$ analytical grade reagent (31, 63, 125 and 250 µg Cu/L), 2 replicates per treatment
Test acceptability criteria	Mean number of egg-masses in the control ≥ 3 per live snail, with mean number of eggs being ≥ 5 per egg-mass. ≥ 87.5% non-immobilised snails in control

metals may be present). Refrigerated samples must be warmed for 2 to 3 hours in an environmental cabinet set at 25 ± 1 °C prior to testing.

Sample pH is measured to establish whether it is within the range for the test (6.0 – 9.0). If pH is outside of this range, a subsample (100 mL for screening test, 500 mL for a full dilution series test) is to be collected for pH adjustment, by the addition of 0.1 M NaOH or HCl as appropriate until pH is 7.0 ± 0.1. Subsequent test design should incorporate parallel pH control treatments to determine pH effect on sample toxicity. De-chlorination (if required) is achieved by the addition of sodium thiosulphate to a subsample at a rate of 6.7 mg/L for every 1 mg/L total chlorine in the sample. Subsequent test design should incorporate parallel sodium thiosulphate control treatments.

3.1.5 Daily test procedure

Day 0

Test setup and test solution preparation

- Test datasheets are prepared for each sample tested.
- Pre-rinsed 600 mL beakers are labelled according to datasheet details, with four replicates for each treatment, labelled A to D.
- An additional 100 mL beaker is labelled for each treatment for the determination of physico-chemical parameters.
- To prepare a standard dilution series from a bulk sample (e.g. an effluent), refer to Table 9 for the volume of sample and modified DMW required. Bulk test solutions are stored in glass Schott bottles.
- 500 mL of each solution is dispensed into the pre-labelled 600 mL beakers (one beaker for each replicate per treatment in the dilution series).
- 50 mL of each solution is dispensed into the pre-labelled 100 mL beaker for determination of physico-chemical parameters.
- All beakers are placed on a plastic tray, covered with BPA-free plastic film and placed in the 25 °C environmental chamber on a 16 hour light: 8 hour dark cycle while reference toxicant test solutions are prepared.

Table 9: Suggested volume required for the preparation of bulk test solutions (at day = 0), based on % dilution of a sample

Concentration (%)	Volume 100% sample (mL)	Volume modified DMW (mL)
DMW (Control)	0	2000
6.3	125	1875
12.5	250	1750
25	500	1500
50	1000	1000
100	2000	0

Reference toxicant preparation

Reference toxicant (positive control) testing with dissolved copper (0.45 µm filtered fraction) is undertaken each time a toxicity test is undertaken and provides a mechanism for checking test precision. Any deviation of a test result from previously established criteria (Appendix A.3) may highlight issues with test organism health, the test conditions or laboratory technician competence. Reference toxicity testing for the snail (*G. georgiana*) test is performed in duplicate with the following concentrations: 0, 31, 63, 125, 250 µg Cu/L.

- A 0.1 g Cu/L stock solution is prepared by adding 0.3932 g of $CuSO_4.5H_2O$ to 1 L deionised water in a 1 L volumetric flask.
- Stock solution is transferred to a Schott bottle and stored in the dark at room temperature until required (shelf life 1 year).
- Each copper test concentration is prepared from the stock solution in separate 1 L volumetric flasks by serial dilution.
- 500 mL of each solution is dispensed into pre-labelled 600 mL beakers, one beaker for each duplicate in the dilution series.

Addition of test organisms, feeding and measurement of physico-chemical parameters

Mature egg-laying snails of length 8 to 12 mm (from tip of shell apex to spire), with no evidence of damage or detachment are selected for testing. These are obtained directly from healthy laboratory aquarium cultures (refer Appendix A.1 for detailed culture development methodology and Appendix A.2 for the *Standard operating procedure for culture maintenance: Gastropod mollusc*, *Glyptophysa georgiana*).

- Snails are detached from the glass walls of the aquaria using a bristle brush or forceps and placed in Petri dishes with a small volume of modified DMW.
- Two snails are randomly assigned to each 600 mL beaker containing test solutions, using forceps or a bristle brush (rinsed with deionised water between replicates).
- One large flake of Sera Vipan food per snail is introduced to each beaker.
- Beakers are randomly arranged onto plastic trays, which are placed in a 25 °C environmental chamber.
- Test solutions are aerated at a rate of one bubble per second using a Pasteur pipette, silicone tubing, gang-valve to regulate air flow and an aquarium pump.
- Each beaker is covered with a polycarbonate disc with aeration fittings inserted through a central hole.
- Time of addition of the first test organism to test solution is noted on the datasheet as t = 0.
- Physico-chemical parameters (temperature, pH dissolved oxygen concentration and electrical conductivity) are measured at test commencement using a multi-parameter digital water quality meter.

Days 1, 2 and 3

Feeding, test solution renewal (if required) and measurement of physico-chemical parameters

- Snails are fed daily with 1 large Sera Vipan flake per surviving snail.
- Uneaten flake from the previous day, faeces and other organic debris is siphoned from the beaker using 5 mm diameter silicone tubing.
- Unattached snails are examined and if no response is observed when gently nudged with a glass probe, they are assumed to be dead and removed from the test beaker.
- In a static renewal regime, test solutions are renewed daily, prior to feeding:
  - A set of fresh test solutions (including reference toxicant solutions) are prepared daily as outlined in *Test setup and test solution preparation* and *Reference toxicant preparation*. Renewal solutions are held in the same environment cabinet as the test vessels, for temperature consistency.
  - Old solutions are to be poured into a corresponding set of labelled beakers to be retained for determination of physico-chemical parameters.
  - Test beakers are rinsed with 50 mL DMW by gentle swirling to dislodge debris and dead snails are removed, taking care not to discard unattached live snails.
  - Each test beaker is filled with the corresponding test solution.
  - Steps outlined for Day 0 are to be repeated with physico-chemical parameters measured in old and residual test solutions and recorded on the corresponding datasheet.

Daily observations

Each day, the following observations are undertaken and recorded on the datasheet:

- Number of alive and dead snails
- Number of egg-masses
- Number of eggs per mass

To distinguish between egg-masses laid on different days, the outline of the egg-mass is circled on the outside of the beaker as it is recorded. In the case of a static renewal regime these observations are to be recorded prior to solution renewals.

Day 4 (Test termination)

Tests are terminated 96 hours from test commencement.

- Aeration system is detached, and test beakers are removed from the environmental cabinet and arranged in order of increasing test concentration.
- The number of surviving snails, egg-masses and number of eggs per mass (for the previous 24 hours) is recorded on the datasheet.
- Physico-chemical parameters (temperature, pH dissolved oxygen concentration and electrical conductivity) of the test solutions are measured and recorded.

3.1.6 Test acceptability criteria

The following criteria are to be met for the test to be considered acceptable:

- ≥ 87.5% non-immobilised snails in the controls
- 3 egg-masses per surviving snail in the controls
- Mean number of eggs per mass ≥ 5 in the controls
- The 96 h EC50 estimate for the copper reference toxicant (positive control) must fall within previously established lower and upper warning limits*
- All physico-chemical parameters are to be maintained throughout the test duration. Specifically, temperature: 25 ± 1 °C, pH range: 7.6 to 8.2 and dissolved oxygen concentration: 90 to 105% saturation (7.4 to 8.6 mg/L).

* The EC50 estimate for the copper reference toxicant must fall within two standard deviations of the running mean as depicted on the *Control Chart for Gastropod Mollusc Test: G. georgiana* (Appendix A.3). If the EC50 estimate for the reference toxicant falls outside of two standard deviations of the running mean, but within three standard deviations, an investigation into the likely cause of departure is to be initiated (e.g. contamination, culture health, culture fatigue or laboratory technician error), which will inform whether the results may be acceptable. However, if the EC50 estimate falls outside of three standard deviations of the running mean, the results are considered unacceptable, and all tests should be repeated. Note: it is expected that testing laboratories will generate their own control chart with their chosen reference toxicant.

3.1.7 Analysis of test data

Test endpoints are calculated primarily on the total number of eggs produced by the surviving snails over the 96 hour exposure period. However, analyses can also be undertaken on the basis of the number of egg-masses produced as a total over the test duration, or over each 24 hour period.

For estimates using statistical approaches assuming normality and homogeneity, data are screened for normality and homogeneity and transformed where necessary. Mean number of eggs produced is determined for each toxicant concentration and controls at the end of the experiment. Standard deviation, standard error and per cent coefficient of variation are calculated for each treatment as an indicator of within-treatment variability. Concentration-response relationships are determined and effect concentrations (ECx), generally EC10 and EC50 estimates, i.e. the concentration which results in a 10% or 50% reduction in egg production in the time prescribed by the test, are calculated with statistical software suitable for the analysis of toxicity data. The No Significant Effect Concentration (NSEC) – the concentration at which the modelled mean response is statistically indistinguishable from the mean control response, may be calculated through methods described by Fisher and Fox (2023) and Fisher et al. (2023). The No Effect concentration (NEC) – the minimum concentration above which an effect is predicted to occur, may be calculated through methods described by Fox et al. (2010).

Resulting chronic effect datasets are suitable for use in the derivation of water quality guideline values (for various levels of aquatic species protection) using the species sensitivity distribution (SSD) approach, the preferred method for guideline derivation for Australia and New Zealand (ANZG 2018; Warne et al. 2025), and ssdtools software (Thorley et al. 2025; Thorley and Schwarz 2018) through its accompanying web-based interface, shinyssdtools software (Dalgarno 2021, 2018; Fox et al. 2023, 2021).

3.2 TEST PROCEDURE: CNIDARIAN, *HYDRA VIRIDISSIMA* (SOUTHWEST AUSTRALIAN ISOLATE) POPULATION GROWTH INHIBITION CHRONIC TOXICITY TEST

Hydra viridissima (Pallas, 1766) is a freshwater cnidarian with a global distribution including many regions of Australia. *H. viridissima* is the only known hydra species that has a permanent symbiotic relationship with the unicellular green alga *Chlorella* sp., which occupies endodermal epithelial cells. The algal symbionts provide nutrients derived from photosynthetic activity and are responsible for the distinct green colouration of this species. *H. viridissima* inhabits ponds, lakes and slow-flowing areas of streams and rivers, where they attach to the substrate, usually vegetation, via a basal disc.

Individuals of the Southwest Australian isolate are typically up to 10 mm in length when fully extended (Figure 9). Hydra are hermaphroditic, with the ability to reproduce sexually or asexually. Laboratory cultures rely on asexual reproduction, where buds are produced along the body wall. Typically, a single bud is produced at a time, although up to two buds have been observed to develop simultaneously in the Southwest Australian isolate. The buds generally detach within a day of development and form an individual hydra. Detached hydra can float for several hours before attaching to a substrate.

3.2.1 Method summary

The following standardised procedure can be used to estimate the chronic toxicity of single chemicals, complex effluents, leachates, receiving waters and elutriates of sediments to the freshwater green hydra, *Hydra viridissima* (Southwest Australian isolate).

The procedure is based on the methodology developed by the Environmental Research Institute of the supervising Scientist (ERISS) in the Northern Territory and described by Riethmuller et al. (2003) and Trenfield et al. (2020) for a Northern Territory isolate of the same species. The original method was modified for the current study for use with hydra isolated from natural waters and the higher conductivity and hardness that reflect broader Southwest Australian conditions. In this test, asexually reproducing (budding) hydra are exposed to the test material for 96 hours, at a range of dilutions, and effect on population growth is assessed (Figure 10). Test design comprises four replicates per treatment, each with ten randomly selected hydroids with a single bud with tentacles. Test animal condition and number of individual hydroids are assessed every 24 hours until the test is terminated at 96 hours.

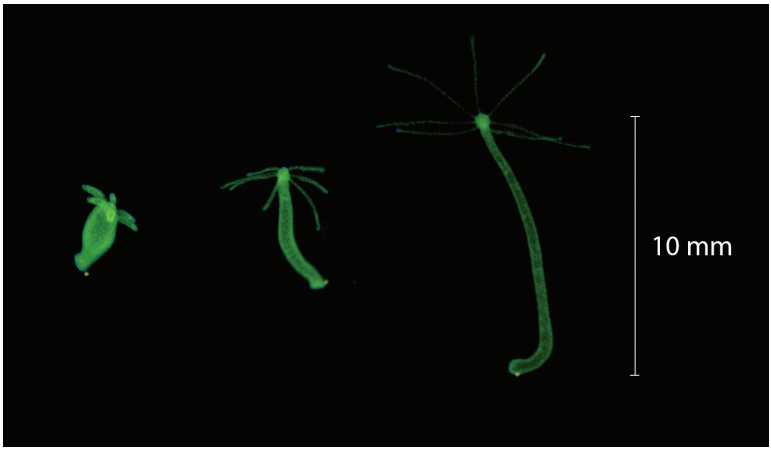


Figure 9: *Hydra viridissima* (Southwest Australian isolate). Photo credit: D. Halliday

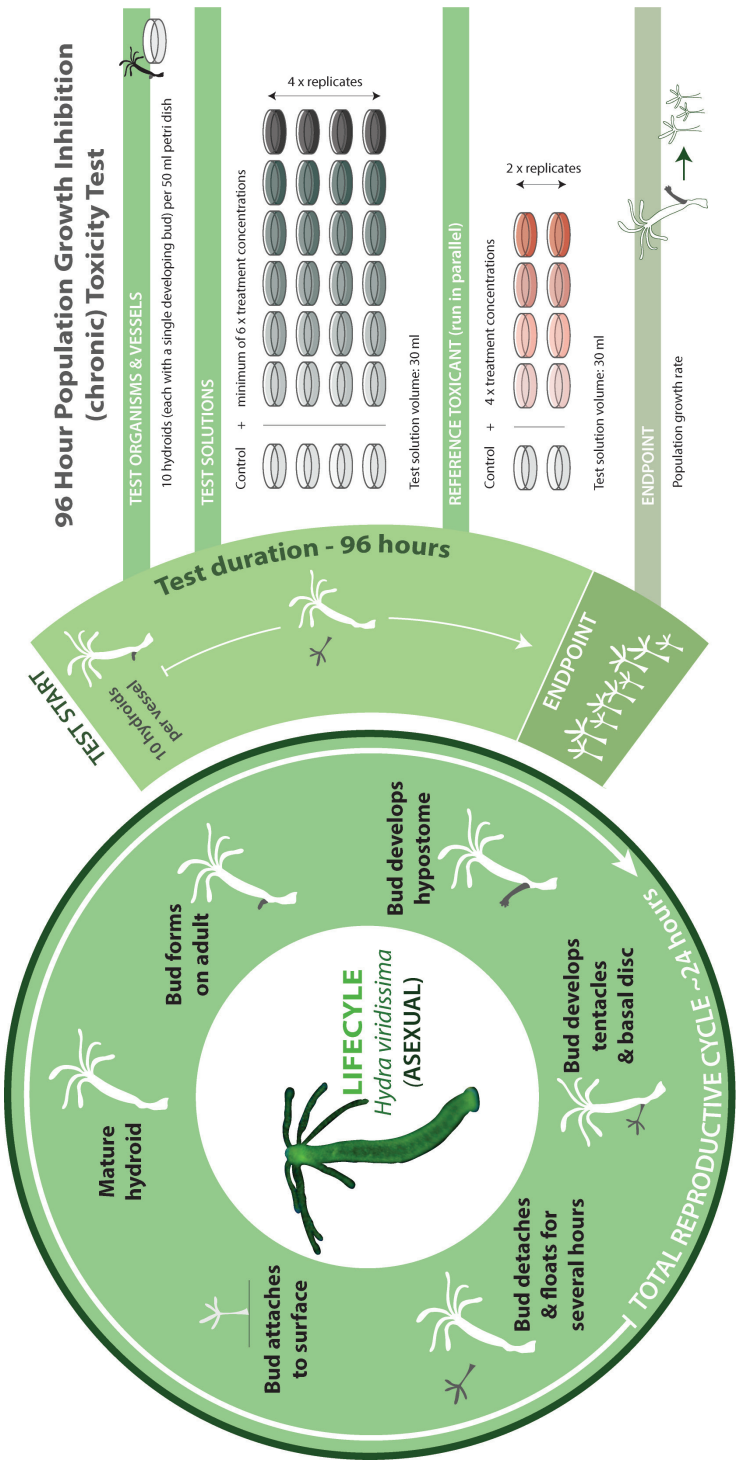


Figure 10: ***Hydra viridissima*** **lifecycle and toxicity test experimental design details**

This procedure describes routine testing. Test conditions and acceptability criteria are provided in Table 10. Modifications may be made to test requirements to suit region or site-specific conditions within the tolerance ranges presented in Table 6 (Section 2).

3.2.2 Organism culture

Cultures of *H. viridissima* used for ecotoxicity testing are maintained in 500 mL (minimum volume) covered glass dishes containing dilute mineral water (DMW) culture media. Culture water is lightly aerated using a Pasteur pipette connected to an aquarium pump airline, and maintained at a temperature of 25 ± 1 °C, pH range of 7.6 to 8.2, dissolved oxygen of 90 to 105% saturation (7.4 to 8.6 mg/L) and a conductivity of 400 ± 80 µS/cm. Cultures are held in either *holding mode* or *build-up* mode. *Build-up mode* should be initiated one week prior to testing to ensure an adequate supply of healthy budding hydra for testing. Healthy cultures will contain a high proportion of individuals with long tentacles, are asexually reproducing and are actively feeding by trapping brine shrimp when supplied. Detailed methodology on culture development and maintenance is provided in Appendix B.1. A Standard operating procedure for culture maintenance for Cnidarian test: *H. viridissima* is provided in Appendix B.2.

Source cultures of *H. viridissima* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Drive, Lane Cove, NSW, Australia, 2066; info@ecotox.com.au.

Table 10: Summary of test conditions and test acceptability criteria for the green hydra, *Hydra viridissima* (Southwest Australian isolate) population growth inhibition (chronic) test

Test parameter	Test Condition
Test species	*Hydra viridissima* (Southwest Australian isolate)
Test type	Static renewal
Test duration	96 hr
Temperature	25 ± 2 °C
Light quality	Cool-white fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16 h light: 8 h dark
Test chamber size	50 mL
Test solution volume	30 mL
Renewal of Test Solutions	Every 24 h
Age of test organisms	Hydroid with a single bud with tentacles
No. of organisms per replicate	10
No. of replicates per treatment	4
No. of organisms per treatment	40
Feeding regime	Fed brine shrimp nauplii daily
Test chamber cleaning	Rinsed daily
Test chamber aeration	None
Dilution water	Modified Dilute Mineral Water (DMW), prepared by mixing 20% Perrier mineral water with deionised water, with GP2 salts
Test concentrations	Minimum of 6 concentrations plus a control
Dilution series	≥ 0.5 dilution series (e.g. 100, 50, 25, 12.5 and 6.25%)
Endpoint	Population growth rate
Reference Toxicant	$CuSO_4.5H_2O$ analytical grade reagent (1.1, 3.3, 10 and 30 µg Cu/L), 2 reps per treatment.
Test acceptability criterion	Mean number of normal hydroids in the control ≥ 20, corresponding to a population growth rate of 0.17 hydra/day

3.2.3 Equipment

- constant temperature chambers with light cycling
- refrigerator
- Pasteur pipettes & bulbs
- pre-prepared Diluent (DMW)
- 50 mL Petri dishes, borosilicate glass or plastic depending on the contaminant(s) being tested
- pH, temperature, conductivity and dissolved oxygen meters (and associated calibration fluids)
- various borosilicate glassware, including 600, 250 and 100 mL beakers, 1 L measuring cylinders, 1 and 2 L screw cap Schott bottles, 50 mL Petri dishes, 1 L volumetric flasks
- autopipettors (range: 10 to 5000 µL) and polyethylene tips
- plastic trays
- washed brine shrimp (*Artemia salina*) nauplii to feed test organisms.

3.2.4 Test preparation

Test diluent and control water

Test diluent and control water is a standard dilute mineral water (DMW): 20% mineral water: 80% deionised water (USEPA 2002a), prepared by mixing 3.2 L deionised water with 800 mL mineral water in a 5 L bottle at least 4 hours prior to test commencement, to ensure dissolved carbon dioxide and pH have reached equilibrium. This is then modified with the addition of GP-2 salts (USEPA 2002b, Appendix F) while stirring with a magnetic stirrer to raise the electrical conductivity to 500 ± 50 µS/cm. Modified DMW is to be used within 4 days.

Note: where a toxicity assessment requires a field-collected diluent, e.g. upstream of a discharge point, the field-sourced diluent may not be optimal with respect to the health of the test organisms, resulting in test acceptability criteria (Table 10) not being met. As such, test design should include negative control treatments using both modified DMW and field-sourced diluents that can be statistically compared.

Sample preparation: effluents, receiving water, ground waters and leachates

Volume of test material (sample) required per treatment is 2 L. Sample may need to be pre-filtered th rough a 60 µm mesh (stainless steel for samples where organic contaminants may be present and nylon where metals may be present). Refrigerated samples are warmed for 2 to 3 hours in an environmental cabinet set at 25 ± 1 ^{o}C prior to testing.

Sample pH is measured to establish whether it is within the range for the test (7.6 to 8.2). If pH is outside of this range, a subsample (100 mL for screening test, 500 mL for a full dilution series test) is to be collected for pH adjustment, by the addition of 0.1 M NaOH or HCl as appropriate until pH is 7.0 ± 0.1. Subsequent test design should incorporate parallel pH control treatments to determine pH effect on sample toxicity. If the samples require dechlorinating, this is achieved by the addition of sodium thiosulphate to a subsample at a rate of 6.7 mg/L for every 1 mg/L total chlorine in the sample. Subsequent test design should incorporate parallel sodium thiosulphate control treatments.

3.2.5 Daily test procedure

Day 0

Test setup and test solution preparation

- Test datasheets are prepared for each sample tested.
- Pre-rinsed 50 mL Petri dishes are labelled according to datasheet details, with four replicates labelled A to D.
- Pre-rinsed 250 mL beakers are also labelled according to datasheet details, with the four replicates labelled A to D.
- To prepare a standard dilution series from a bulk sample (e.g. for an effluent), refer to Table 11 for the volume of sample and modified DMW required.
- 200 mL of each test solution is dispensed into pre-labelled 250 mL beakers (one beaker for each replicate per treatment in the dilution series).
- 30 mL of each solution is dispensed into the pre-labelled 50 mL Petri dishes for each treatment.
- pH, conductivity and dissolved oxygen is measured in each 250 mL beaker and values recorded.
- All Petri dishes are placed on a plastic tray, covered with BPA-free plastic film and placed in the 25 °C environmental chamber while reference toxicant test solutions are prepared.
- Remaining test solutions are stored in Schott bottles in the refrigerator for renewing the test solutions at 24, 48 and 72 hours. Note: fresh test solutions are to be prepared each day if the bulk stock solutions are unlikely to be stable under refrigeration.

Reference toxicant preparation

Reference toxicant (positive control) testing with dissolved copper (0.45 µm filtered fraction) is undertaken each time a toxicity test is undertaken and provides a mechanism for checking test precision. Any deviation of a test result from previously established criteria may highlight issues with test organism health, the test conditions or laboratory technician competence. Reference toxicity testing is performed in duplicate with the following concentrations: 0, 1.1, 3.3, 10, 30 µg Cu/L.

- A 0.1 g Cu/L stock solution is prepared by adding 0.3932 g of $CuSO_4.5H_2O$ to 1 L deionised water in a 1 L volumetric flask.
- Stock solution is transferred to a Schott bottle and stored in the dark at room temperature until required for use (shelf life of 1 year).

Table 11: Suggested volume required for the preparation of bulk test solutions (at day =0), based on % dilution of a sample

Concentration (%)	Volume 100% sample (mL)	Volume modified DMW (mL)
DMW (Control)	0	2000
6.3	125	1875
12.5	250	1750
25	500	1500
50	1000	1000
100	2000	0

- Each copper test concentration is to be prepared from the stock solution in separate 200 mL volumetric flasks by serial dilution.
- 30 mL of each solution is dispensed into pre-labelled Petri dishes, one for each duplicate in the dilution series.

Addition of test organisms, feeding and measurement of physico-chemical parameters

Hydroids used for testing must have a single bud with tentacles. These are obtained directly from healthy laboratory mass cultures (refer Appendix B.1 for detailed culture development methodology and Appendix B.2 for the Standard operating procedure for culture maintenance for Cnidarian test: *Hydra viridissima*).

- Approximately 30 mL of diluent water is transferred to a Petri dish.
- Vessels containing mass cultures are observed under the dissecting microscope and hydroids exhibiting a single bud with tentacles are collected using a Pasteur pipette and transferred to the Petri dish. Note: the tentacles should be present only as bumps on the bud and the bud must not appear ready to detach from the main stem of the hydroid.
- Once enough hydroids have been collected for the test, the culture tray is returned to the environment cabinet.
- Individual Petri dishes containing test solutions are randomly arranged onto plastic trays before seeding the test, to minimise disturbance of test organisms.
- The Petri dish containing the isolated hydroids is placed under the dissecting microscope and 10 hydroids are transferred to each replicate vessel for each treatment, ensuring the pipette does not contact test solutions.
- Once each Petri dish contains 10 organisms, the dishes are covered, and the trays are transferred to the environment cabinet set at 25 ^{o}C and 16 hour light: 8 hour dark cycle.
- Time of addition of the first test organism to test solution is noted on the datasheet as t=0.
- Physico-chemical parameters (temperature, pH dissolved oxygen and electrical conductivity) are to be recorded at test commencement.

Days 1, 2 and 3

Feeding, test solution renewal and measurement of physico-chemical parameters

Hydroids are fed daily with brine shrimp nauplii and test solutions are renewed daily according to the following procedure:

- A set of fresh test solutions (including reference toxicant solutions) are prepared daily as outlined in *Test setup and test solution preparation* and *Reference toxicant preparation.* Renewal solutions are held in the same environment cabinet as the test vessels, for temperature consistency.
- Trays holding the test Petri dishes are removed from the environment cabinet and each hydroid is fed using a Pasteur pipette, by gently ejecting brine shrimp in its vicinity so that some shrimp become caught in the tentacles (detailed methodology relating to food preparation is provided in Appendix B.2).
- Once brine shrimp have been provided to all hydra, the trays are returned to the environment cabinet, where feeding is allowed to continue for up to 6 hours.

- After between 2 and 6 hours from feeding, trays holding the 100 mL beakers of test solutions and the Petri dishes containing test hydra are removed from the environment cabinet and arranged in order of increasing concentration and daily observations (see following section) are undertaken.
- Solutions are then renewed by swirling each Petri dish to dislodge uneaten brine shrimp and regurgitated food, then solution is transferred into a second (cleaning) Petri dish.
- 5 mL of fresh solution from the corresponding 100 mL beaker is used to carefully rinse the test dish, then discarded into the cleaning dish.
- Petri dish solutions are restocked and any hydra that become dislodged during cleaning are transferred from the cleaning dish back to the test dish.
- Dead hydra are removed.
- Old solution in the cleaning dish is retained for determining physico-chemical parameters.
- Test dishes are re-randomised and returned to the environment cabinet.

Daily observations

Each day the number of living and normally developed hydroids (green, tentacles, no abnormalities) is recorded for each test vessel. Physico-chemical parameters (temperature, pH dissolved oxygen and electrical conductivity) in both old and residual test solutions are measured and recorded.

Day 4 (Test termination)

Tests are terminated 96 hours from test commencement.

- Petri dishes are removed from the environment cabinet and arranged in order of increasing test concentration.
- The number of normally developed hydra in each dish is recorded on the datasheet.
- Physico-chemical parameters (temperature, pH, dissolved oxygen and electrical conductivity) of the test solutions are measured and recorded on the datasheet.

3.2.6 Test acceptability criteria

A test is considered acceptable if the following criteria are met:

- Mean number of normal hydra ≥ 20 (growth rate of 0.17 hydra/day) in the controls.
- The 96 hour EC50 estimate for the copper reference toxicant must fall within lower and upper warning limits*
- All physico-chemical parameters are to be maintained throughout the test duration. Specifically, temperature: 25 ± 1 °C, pH range: 7.6 to 8.2 and dissolved oxygen concentration: 90 to 105% saturation (7.4 to 8.6 mg/L).

*Lower and upper warning limits are calculated as two standard deviations of the mean and are used to provide a warning that test organisms for any given test are not responding to the reference toxicant as expected. Specifically for this test, if the EC50 estimate for the reference toxicant falls between two and three standard deviations of the running mean, as depicted on the *Control Chart for Cnidarian Test: H. viridissima* (Appendix B.3), an investigation into the likely cause of departure is to be initiated (including contamination, culture health, culture fatigue, laboratory technician error), which will inform whether the data are acceptable. However, if the EC50 estimate falls outside of

three standard deviations of the running mean, the results are considered unacceptable, and all tests should be repeated. Note: it is expected that testing laboratories will generate their own control chart with their chosen reference toxicant.

3.2.7 Analysis of test data

The data from the hydra population growth test are used to generate population growth rates for each test concentration and control. Population growth rate is determined using the formula:

$$\frac{\ln(\#\text{ hydra at Day } 4 \pm 0.00001) - \ln(\#\text{ hydra at Day } 0 \pm 0.00001)}{4}$$

For estimates using statistical approaches assuming normality and homogeneity, data are screened for normality and homogeneity and transformed where necessary. Mean growth rate is determined for each treatment at the end of the experiment. Standard deviation, standard error and per cent coefficient of variation are calculated for each treatment as an indicator of within-treatment variability. Concentration-response relationships are determined and effect concentrations (EC_x), generally EC10 and EC50 estimates, i.e. the concentration which results in a 10% or 50% effect in the time prescribed by the test, are calculated with statistical software suitable for the analysis of toxicity data. The No Significant Effect Concentration (NSEC) – the concentration at which the modelled mean response is statistically indistinguishable from the mean control response, may be calculated through methods described by Fisher and Fox (2023) and Fisher et al. (2023). The No Effect concentration (NEC) – the minimum concentration above which an effect is predicted to occur, may be calculated through methods described by Fox et al. (2010).

Resulting chronic effect datasets are suitable for use in the derivation of water quality guideline values (for various levels of aquatic species protection) using the species sensitivity distribution (SSD) approach, the preferred method for guideline derivation for Australia and New Zealand (ANZG 2018; Warne et al. 2025), and ssdtools software (Thorley et al. 2025; Thorley and Schwarz 2018) through its accompanying web-based interface, shinyssdtools software (Dalgarno 2021, 2018; Fox et al. 2023, 2021).

3.3 TEST PROCEDURE: CLADOCERAN, *SIMOCEPHALUS EXSPINOSUS* (SOUTHWEST AUSTRALIAN ISOLATE) PARTIAL LIFECYCLE CHRONIC TOXICITY TEST

Simocephalus exspinosus De Geer 1778 (Figure 11) is a species of water flea previously recorded in NSW, Tasmania, and South Australia. It was first identified as *Simocephalus australiensis* Dana 1852 and as *Simocephalus exspinosus australiensis* (Smirnov and Timms 1983). *S. exspinosus* has been reported in temporary pools in Europe (Crosetti and Margaritora 1987) and slow-flowing parts of creeks and rivers (current study). *S. exspinosus* reproduces both asexually (by parthenogenetic subitaneous reproduction), as shown in Figure 12, and sexually (by sexual ephippial reproduction).

In the current study, females with ephippia (resting eggs) have not been observed in culture but mature females with neonates were observed to inhabit the base of culture vessels suggesting that this species may be epifaunal in nature. The Southwest Australian isolate has a carapace of approximately 2.5 mm in length (females). Males (although not observed in culture) are expected to be half the size of females of a similar age (Smirnov and Timms 1983).

Like other cladocerans used for toxicity testing (*Ceriodaphnia* cf. *dubia*, *Daphnia carinata* and *Moina australiensis)*, *S. exspinosus* appears to be photosensitive and will migrate away from a strong light source such as a fluorescent light that might be used to aid capturing organisms for culturing or isolating for testing.

3.3.1 Method Summary

The following standardised procedure can be used to estimate the chronic toxicity of single chemicals, complex effluents, leachates, receiving waters and elutriates of sediments to the freshwater cladoceran, *S. exspinosus* (Southwest Australian isolate).

This procedure is based on the methodology developed by the USEPA (2002b) for *Ceriodaphnia* cf. *dubia* (Method 1002.0), which was adapted for use in Australia with the NSW sourced *Ceriodaphnia* cf. *dubia* (the Sydney clone) by Bailey et al. (2000). This methodology has been adapted further in

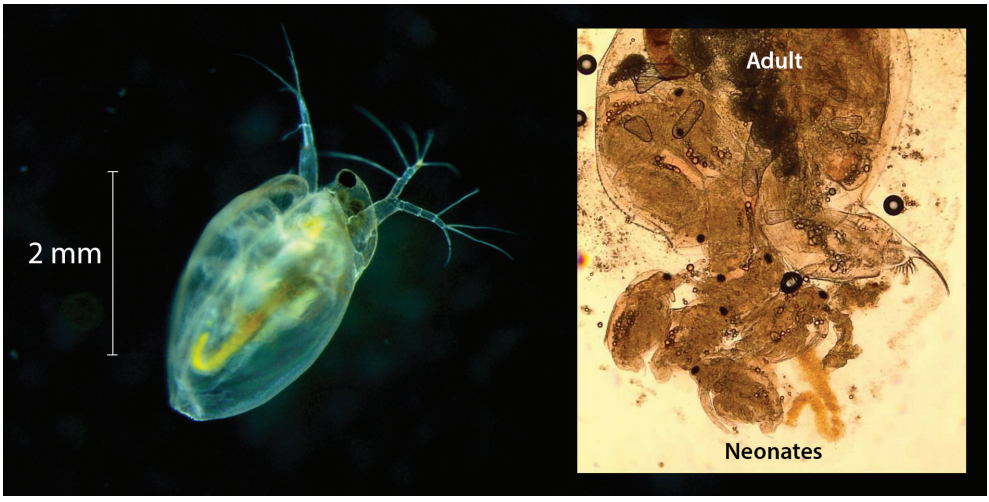


Figure 11: Mature female *Simocephalus exspinosus* (Southwest Australian isolate). Photo credit: D. Halliday. **Inset shows neonates.** Photo credit: R. Shiel.

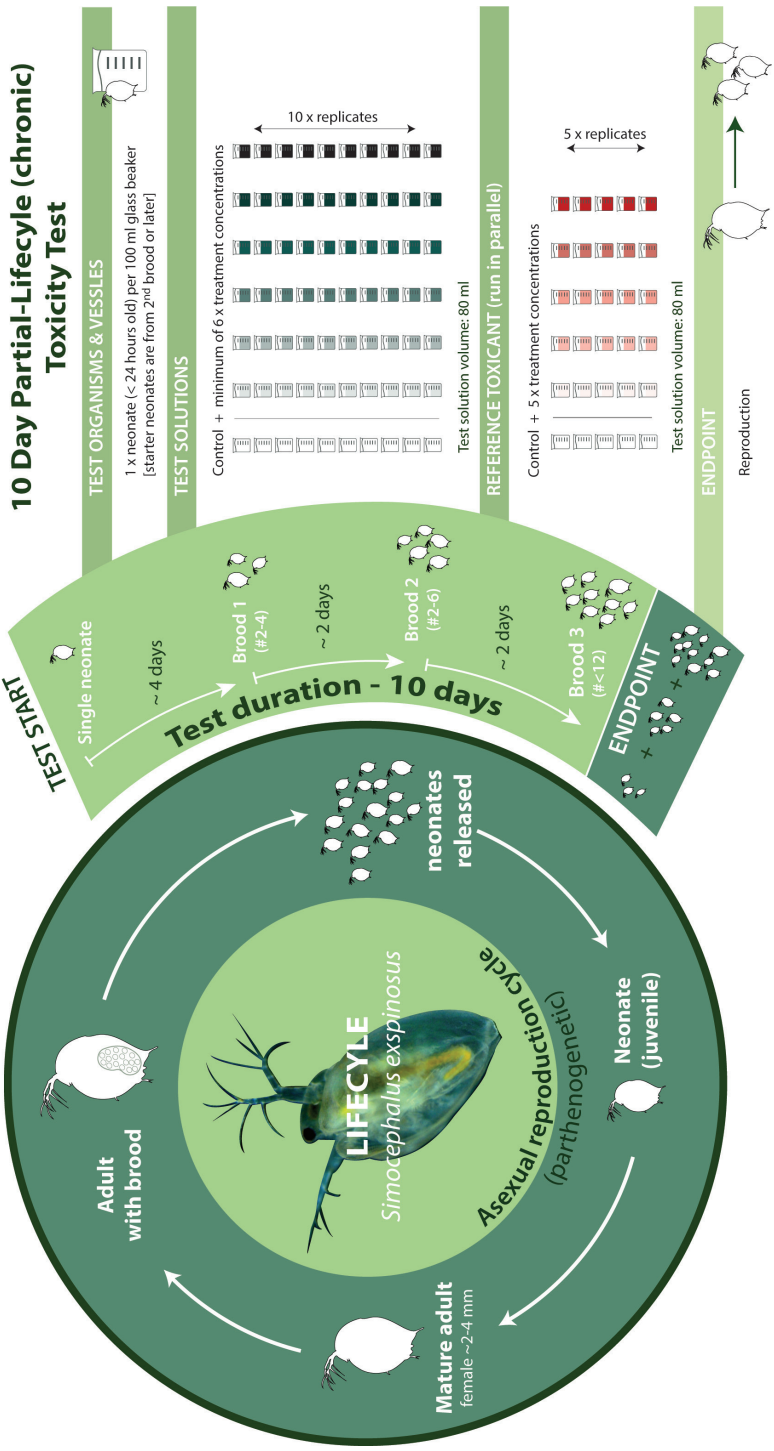


Figure 12: *Simocephalus exspinosus* lifecycle and toxicity test experimental design details

the current study for use with *S. exspinosus* (Southwest Australian isolate) and Southwest Australian conditions. In this test, *S. exspinosus* neonates, less than 24 hours old, are exposed to the test material at a range of dilutions in a static renewal system, until 60% of non-immobilised control organisms have produced at least 15 offspring over 3 broods, within 10 days (Figure 12).

Test design comprises ten replicates per treatment, each with a single, randomly selected neonate. Effects on survival and reproduction are assessed every 24 hours until test termination (maximum of ten days).

This procedure describes routine testing. Test conditions and acceptability criteria are provided in Table 12. Modifications may be made to test requirements to suit region or site-specific conditions within the tolerance ranges presented in Table 6 (Section 2).

Table 12: Summary of test conditions and test acceptability criteria for the *Simocephalus exspinosus* partial lifecycle (chronic) toxicity test

Test parameter	Test Condition
Test species	*Simocephalus exspinosus* (Southwest Australian isolate)
Test type	Static renewal
Test duration	Until 60% of non-immobilised control organisms have 3 broods (maximum 10 days)
Temperature	25 ± 1 °C
Light quality	cool-white, fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16 h light: 8 h dark
Test Chamber size	100 mL
Test solution volume	80 mL
Renewal of Test Solutions	24 h or 48 h
Age of test organisms	Less than 24 h old
No. of organisms per replicate	1
No. of replicates per treatment	10
No. of organisms per treatment	10
Feeding regime	*Raphidocelis subcapitata* and freeze dried *Dunaliella salina*, daily
Test chamber aeration	None
Dilution water	Dilute Mineral Water (DMW), prepared by mixing 20% Perrier mineral water with deionised water, with vitamin B12 and selenium supplements
Test concentrations	Minimum of 6 concentration plus a control
Dilution series	≥ 0.5 dilution series (e.g. 100, 50, 25, 12.5 and 6.25%)
Endpoint	Immobilisation* and reproduction
Reference Toxicant	$CuSO_4.5H_2O$ analytical grade reagent (6.3, 12.5, 25, 50 and 100 µg Cu/L), 5 reps per treatment.
Test acceptability criterion	≥ 80% non-immobilised in controls. An average of ≥ 15 young per non-immobilised female in the controls. 60% of non-immobilised control organisms must produce ≥ 3 broods.

** for the purpose of the test, immobilisation is recorded as an indicator of mortality.*

3.3.2 Organism culture

Cultures of *S. exspinosus* used for ecotoxicity testing are maintained in glass vessels containing DMW at a temperature of 25 ± 1 °C, pH range of 7.8 to 8.3, dissolved oxygen of 90 to 105% saturation (7.4 to 8.6 mg/L) and a conductivity of 180 ± 20 µS/cm. Cultures are maintained in the environment cabinet with a lighting regime of 16 hour light: 8 hour dark (800 ± 160 Lux).

Health of the cultures is critical to the success of tests. The following criteria should be met to ensure healthy test organisms:

- Broodstock mortality < 20% in the 7 day period prior to the use of neonates for testing.
- The first brood of neonates should be produced by the end of Day 5 (but normally by Day 4).
- > 6 neonates should be produced in at least one brood (broodstock must be isolated to determine this). In a beaker of 20 producing females, there should be > 120 neonates.
- Males and ephippeal females should be absent in the broodstock since their presence is considered an indicator of culture stress.
- Cladocerans should show no signs of lethargy or paleness.

Detailed methodology on culture development and maintenance is provided in Appendix C.1 and a Standard operating procedure for culture maintenance for Cladoceran test: *Simocephalus exspinosus* (Southwest Australian isolate) is provided in Appendix C.2.

Source cultures of *S. exspinosus* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Drive, Lane Cove, NSW, Australia, 2066; info@ecotox.com.au.

3.3.3 Equipment

- constant temperature chambers with light cycling
- Pasteur pipettes & bulbs
- pre-prepared Diluent (Dilute Mineral Water, DMW)
- 100 mL borosilicate glass or plastic squat form beakers (test vessels), depending on contaminant(s) being tested
- pH, Temperature, Conductivity and Dissolved Oxygen meters and calibration fluids
- various borosilicate glassware, including 600 mL and 250 mL beakers, 1 L and 250 mL measuring cylinders, 1 and 3 L Scott (screwcap) bottles
- *S. exspinosus* neonates < 24 hours old from Mass Cultures
- *Raphidocelis subcapitata* food stock (available from the CSIRO Australian National Algae Culture Collection: https://www.csiro.au/en/about/facilities-collections/collections/anacc/australian-national-algae-supply-service)
- *Dunaliella salina* food stock (available from Nutrakol: https://nutrakol.com/)
- autopipettors (10 to 5000 µL) and polyethylene tips
- plastic trays
- BPA-free plastic film.

3.3.4 Test preparation

Test diluent and control water

Test diluent and control water is a standard dilute mineral water (DMW): 20% mineral water: 80% deionised water (USEPA 2002a), prepared at least 4 hours prior to test commencement, to ensure dissolved carbon dioxide and pH have reached equilibrium. This is then modified by adding vitamin B12 and selenium supplement. Vitamin B12 supplement stock solution (20 mg/L) is prepared by adding 0.005 g of vitamin B12 to 250 mL of polished (all impurities and contaminants removed) water. The solution is transferred to a 250 mL Schott bottle for storage at 4 °C for up to three months. Selenium stock solution (100 mg/L) is prepared by adding 0.1109 g of Na_2SeO_3 to 500 mL of polished water. This stock is not to be added directly to DMW. Instead, a working selenium supplement stock (4 mg Se/L) is prepared by adding 10 mL of the 100 mg/L selenium stock to 240 mL deionised water. The working selenium stock solution is transferred to a 250 mL Schott bottle for storage at 4 °C for up to three months.

Note: where a toxicity assessment requires a field-collected diluent, e.g. upstream of a discharge point, the field-sourced diluent may not be optimal with respect to the health of the test organisms, resulting in test acceptability criteria (Table 6) not being met. As such, test design should include negative control treatments using both modified DMW and field-sourced diluents that can be statistically compared.

Sample preparation: effluents, receiving water, ground waters and leachates

Volume of test material (sample) required per treatment is 2 L. Sample may need to be pre-filtered through a 60 µm mesh (stainless steel for samples where organic contaminants may be present and nylon where metals may be present). Refrigerated sample must be warmed for 2 to 3 hours in an environmental cabinet set at 25 ± 1 °C prior to testing.

Sample pH is measured to establish whether it is within the range for the test (6.0 – 9.0). If pH is outside of this range, a subsample (100 mL for screening test, 500 mL for a full dilution series test) is to be collected for pH adjustment, by the addition of 0.1 M NaOH or HCl as appropriate until pH is 7.0 ± 0.1. Subsequent test design should incorporate pH control treatments run in parallel to determine pH effect on sample toxicity. If the samples require dechlorination, this can be achieved by the addition of sodium thiosulphate to a subsample at a rate of 6.7 mg/L for every 1 mg/L total chlorine in the sample and subsequent test design should incorporate sodium thiosulphate controls.

Samples with high suspended solids concentration may exhibit a reduction in reproduction compared with control treatments due to *S. exspinosus* consuming suspended solids with food, resulting in a relatively poor dietary intake. Suspended solids may also adhere to gill filaments or interfere with feeding processes. As such, visibly turbid samples should be centrifuged to reduce suspended solids concentration and tested in parallel with the uncentrifuged sample.

3.3.5 Daily test procedure

Day 0
Test setup and test solution preparation

- Test datasheets are prepared for each sample tested.
- Pre-rinsed 100 mL beakers are labelled according to datasheet details, with the ten replicates labelled A to J.
- An additional 100 mL beaker is labelled for each treatment to determine physico-chemical parameters.

- Bulk solutions are prepared (Table 13), allowing for daily renewal of test solutions over a ten-day period (unless bulk solutions are unlikely to be stable under refrigeration, in which case solutions should be prepared daily).
- 80 mL of each solution is dispensed into pre-labelled 100 mL beakers, one for each replicate in the dilution series plus the additional beaker for physico-chemical parameters.
- Remaining bulk solution is stored in a refrigerator.

Table 13: Suggested volume required for the preparation of bulk test solutions (at day =0), based on % dilution of a sample

Concentration (%)	Volume 100% sample (L)	Volume modified DMW (L)
DMW (Control)	0	10
6.3	0.63	9.38
12.5	1.25	8.75
25	2.5	7.5
50	5	5
100	10	0

Reference toxicant preparation

Reference toxicant (positive control) testing with dissolved copper (0.45 µm filtered fraction) is undertaken each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established criteria may highlight issues with test organism health, the test conditions or laboratory technician competence. Reference toxicity testing is undertaken with five replicates of each concentration: 0, 6.3, 12.5, 25, 50, 100 µg Cu/L.

- A 0.1 g Cu/L stock solution is prepared by adding 0.3932 g of $CuSO_4.5H_2O$ to 1 L deionised water in a 1 L volumetric flask. Stock solution is transferred to a Schott bottle and stored in the dark at room temperature until required for use.
- Pre-rinsed 100 mL beakers are labelled with the 6 test concentrations, indicating the replicate identity.
- Reference toxicant bulk test solutions (allowing daily renewal for ten days) are prepared in 2 L glass bottles (Table 14) and stored in the refrigerator.

Table 14: Preparation of bulk reference toxicant test solutions in DMW from 0.1 µg Cu/L stock solution

Concentration (µg Cu/L)	Volume of 0.1 µg Cu/L stock (mL)	Volume DMW (mL)
DMW (Control)	0	4000
6.3	0.25	1999.75
12.5	0.5	1999.5
25	1	1999
50	2	1998
100	4	1996

- 80 mL of each solution is dispensed into pre-labelled 100 mL beakers, one beaker for each of five replicates in the dilution series.
- Remaining bulk reference toxicant solutions are stored in the refrigerator.

Addition of test organisms, feeding and measurement of physico-chemical parameters

Neonates (< 24 hours) are used for testing and are obtained from healthy mass aquarium cultures (refer Appendix C1 for culture development and maintenance).

- While examining under a dissecting microscope, neonates are isolated from healthy cultures using a Pasteur pipette and transferred to a 250 mL beaker containing DMW, ensuring that they are released below the surface of the water.
- Again, while examining under a dissecting microscope, using a clean pipette for each treatment and control, one neonate is randomly assigned to each 100 mL test vessel containing 80 mL test solution, ensuring minimal DMW is transferred with each neonate.
- The end of the pipette is cleaned with blotting paper after each addition to prevent cross-contamination.
- Food (*R. subcapitata*) is added to each 100 mL test vessel to achieve a suspension of 200,000 cells per mL along with *D. salina* at a rate of 12.5 g/L.
- Test vessels are randomly assigned to a tray, covered with BPA-free plastic film and transferred to the environment cabinet.
- The time of the addition of the first animals to the test solutions is noted as t = 0.
- Dissolved oxygen concentration, conductivity and pH are measured in the designated beakers and recorded.

Days 1 - 10

Test solution renewal, feeding and measurement of physico-chemical parameters

Test solutions are renewed every 1 to 2 days depending on the stability of the test material. Test organisms are fed daily, and test data is recorded daily.

- Fresh test solutions are prepared in 1 L glass beakers and placed in the 25 °C environmental cabinet for 2 hours to ensure a homogeneous temperature between old and new solutions.
- *R. subcapitata* suspension is added to each vessel containing fresh solutions to achieve 200,000 cells per mL. Freeze-dried *D. salina* is added to each vessel at the rate of 12.5 g/L.
- Prior to renewing solutions, the daily observations (refer following section) are recorded on the datasheet.
- Test solutions are renewed one at a time by first removing the neonates from old test solutions using a Pasteur pipette.
- The parental organism is then drawn into the Pasteur pipette and placed horizontally on the pipette rack.
- The contents of the beaker are placed into the corresponding vessel for the old test solutions
- The bottom of the original beaker is rinsed using a wash bottle containing deionised water and the rinsate is poured into a waste beaker.

- Test solution is replaced by pouring 80 mL from the fresh 1 L beaker for that treatment (concentration) into the test beaker.
- The parental organism is then returned to the 100 mL test beaker, now containing the fresh solution, by gently ejecting below the surface. Any organisms damaged or dropped are discarded and noted on the data sheet.
- Dissolved oxygen concentrations, pH, temperature and conductivity are recorded in the old and new solutions.

Daily observations

Each day the following observations are undertaken and recorded on the datasheet:

- whether parental animal is alive, dead or showing signs of stress
- number of neonates
- number of aborted eggs or embryos
- presence of any males or females carrying ephippia (resting eggs) – refer USEPA (1993) for guidance on distinguishing between parthenogenic females with eggs in the brood pouch, females carrying ephippia, and males.

Test Termination

Tests are terminated when at least 60% of the non-immobilised control organisms have produced at least three broods, either by day 10 or 11. All observations on test organism immobilisation should occur within 2 hours due to the rapid rate of development of *S. exspinosus.*

- The same data are recorded as for the *Daily Observations.*
- Any test organism observed not to have produced young throughout the duration of the test is to be examined under the microscope to determine whether it is male. This is noted on the corresponding datasheet.
- Dissolved oxygen concentrations, pH, temperature and conductivity are recorded.

3.3.6 Test acceptability criteria

The following criteria are to be met for the test to be considered acceptable:

- ≥ 80% non-immobilised control organisms
- Mean number of offspring per non-immobilised female in controls ≥ 15
- 60% of non-immobilised control organisms must produce ≥ 3 broods
- The 96 hour EC50 estimate for the copper reference toxicant (positive control) must fall within previously established lower and upper warning limits.*
- All physico-chemical parameters are to be maintained throughout the test duration. Specifically, temperature of 25 ± 1 °C, pH range 7.8 to 8.3, dissolved oxygen concentration of 90 to 105% saturation (7.4 to 8.6 mg/L) and a conductivity of 180 ± 50 µS/cm.

* The IC50 estimate for the copper reference toxicant must fall within two standard deviations of the running mean as depicted on the *Control Chart for Cladoceran Test: S. exspinosus* (Appendix C3). If the IC50 estimate for the reference toxicant falls outside of two standard deviations of the running mean, but within 3 standard deviations, an investigation into the likely cause of departure is to be

initiated (e.g. contamination, culture health, culture fatigue or laboratory technician error), which will inform whether the results may be acceptable. However, if the EC50 estimate falls outside of three standard deviations of the running mean, all tests should be repeated. Note: it is expected that testing laboratories will generate their own control chart with their chosen reference toxicant.

3.3.7 Analysis of test data

Neonates that were either missing or accidentally injured or killed as a result of handling are deducted from the total number of organisms considered for that treatment. Otherwise, any missing animals are considered to be mortalities as a result of exposure to the test solution.

Test endpoints are calculated for the *S. exspinosus* test on survival and reproduction test data. For estimates using statistical approaches assuming normality and homogeneity, data are screened for normality and homogeneity and transformed where necessary. Mean number of live young produced for each adult female is determined for each treatment at either the time of death of the adult or at the end of the experiment. Standard deviation, standard error and per cent coefficient of variation are calculated for each treatment as an indicator of within-treatment variability. Concentration-response relationships are determined and effect concentrations (EC_x), generally EC10 and EC50 estimates, i.e. the concentration which results in a 10% or 50% effect in the time prescribed by the test, are calculated with statistical software suitable for the analysis of toxicity data. The No Significant Effect Concentration (NSEC) – the concentration at which the modelled mean response is statistically indistinguishable from the mean control response, may be calculated through methods described by Fisher and Fox (2023) and Fisher et al. (2023). The No Effect concentration (NEC) – the minimum concentration above which an effect is predicted to occur, may be calculated through methods described by Fox et al. (2010).

Resulting chronic effect datasets are suitable for use in the derivation of water quality guideline values (for various levels of aquatic species protection) using the species sensitivity distribution (SSD) approach, the preferred method for guideline derivation for Australia and New Zealand (ANZG 2018; Warne et al. 2025), and ssdtools software (Thorley et al. 2025; Thorley and Schwarz 2018) through its accompanying web-based interface, shinyssdtools software (Dalgarno 2021, 2018; Fox et al. 2023, 2021).

3.4 TEST PROCEDURE: CLADOCERAN, *CERIODAPHNIA* CF. *DUBIA* (SOUTHWEST AUSTRALIAN ISOLATE) PARTIAL LIFECYCLE CHRONIC TOXICITY TEST

Ceriodaphnia cf. *dubia* Richard, 1894 (Figure 13) is a species of water flea with global distribution, although prior to this study its known distribution within Australia was limited to inland waters of Queensland, New South Wales and Victoria. The Southwest Australian isolate has a carapace of approximately 1 mm length, similar to *C.* cf. *dubia* (Sydney isolate) from Lake Parramatta in NSW (in culture at other ecotoxicity testing facilities in Australia). However, the Southwest Australian isolate exhibits some distinct differences when compared to the Sydney isolate with respect to reproductive output. In the laboratory under optimal conditions, the Sydney isolate typically produces 3 broods of neonates over a 7 to 8 day period, with the first brood released by day 3 to 4 (Bailey et al. 2000). In contrast, the Southwest Australian isolate requires up to 10 days to produce 3 broods of neonates under similar conditions, with greater variability in brood size. As such standard methodologies have been adapted for the Southwest Australian test to reflect this apparent physiological difference. *C.* cf. *dubia* (Southwest Australian isolate) was observed to occupy the water column of the littoral zone of rivers and creeks (current study). It is able to reproduce both asexually (by parthenogenetic subitaneous reproduction), as shown in Figure 14, and sexually (by sexual ephippial reproduction).

Male *C.* cf. *dubia* (Southwest Australian isolate) have not been observed in culture but are expected to be smaller than females of a similar age and with a more upright swimming motion based on observations of the Sydney isolate. Females of the Southwest Australian isolate with ephippia (resting eggs) have also not been observed in culture. Mature females observed in culture in the current study were observed throughout the water column of a 600 mL mass culture vessel, whereas the neonates generally inhabited the bottom of the water column.

Like other cladocerans used for toxicity testing (*S. exspinosus*, *Daphnia carinata* and *Moina australienis)*, *C.* cf. *dubia* (Southwest Australian isolate) appears to be photosensitive and will migrate away from a strong light source such as a fluorescent light that might be used to aid capturing organisms for culturing or isolating for testing.

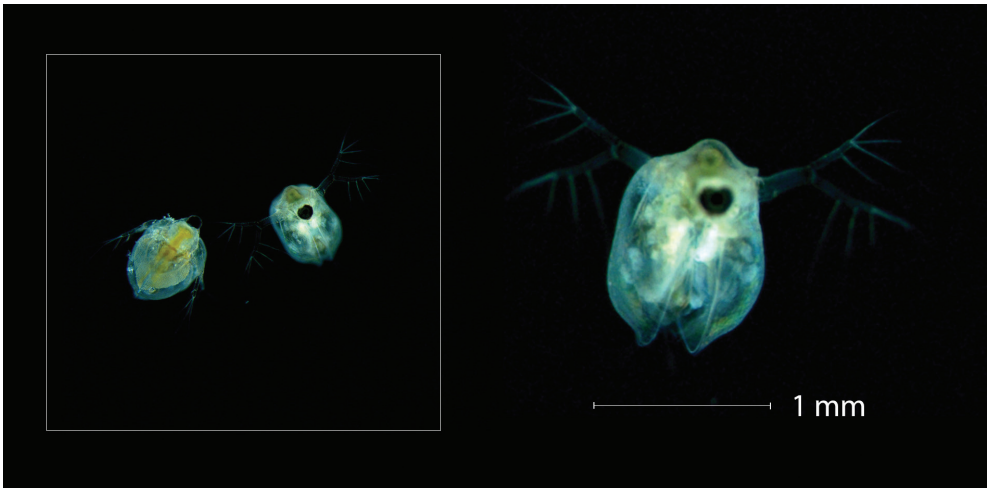


Figure 13: Mature female *Ceriodaphnia* cf. *dubia* (Southwest Australian isolate).
Photo credit: D. Halliday

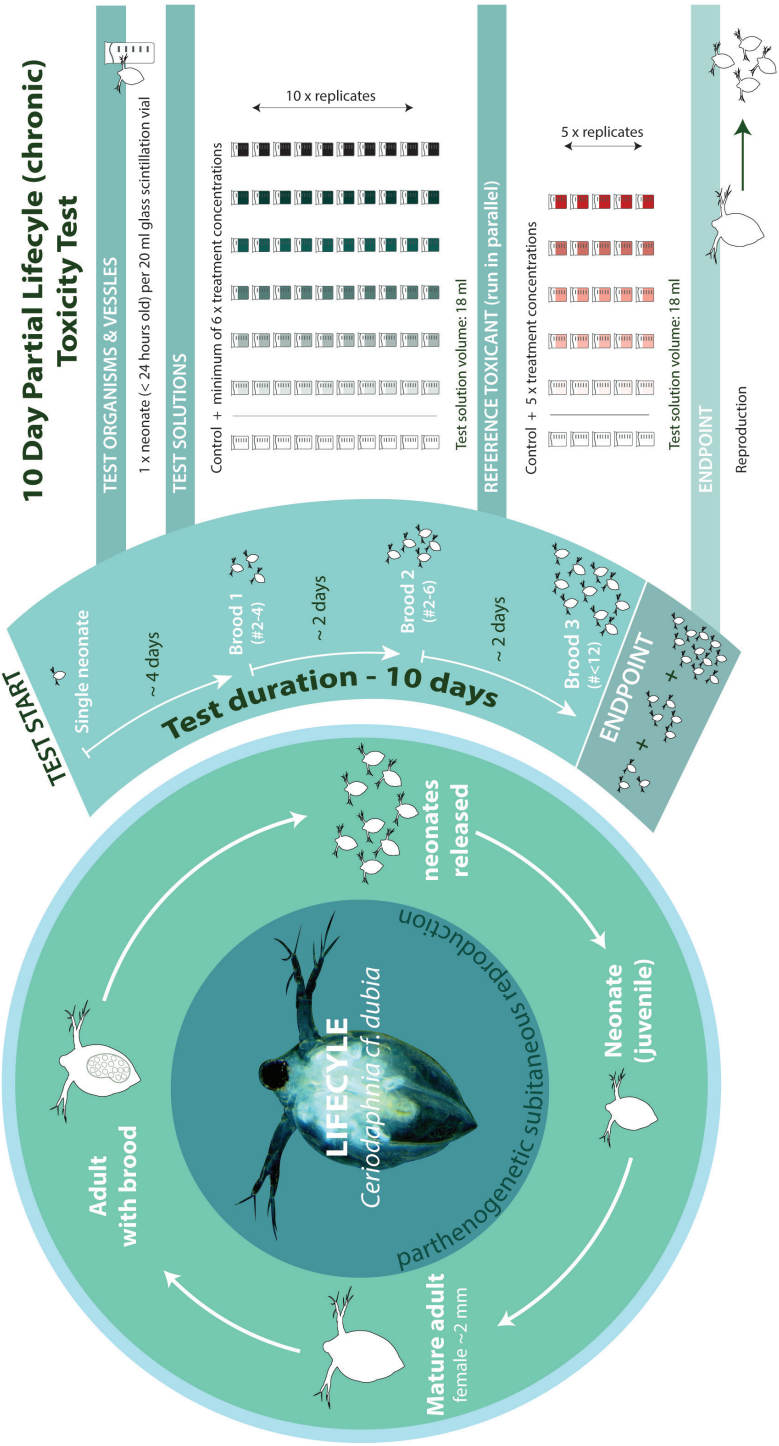


Figure 14: *Ceriodaphnia* cf. *dubia* lifecycle and toxicity test experimental design details

3.4.1 Method Summary

The following standardised procedure can be used to estimate the chronic toxicity of single chemicals, complex effluents, leachates, receiving waters and elutriates of sediments to the freshwater cladoceran, *C.* cf. *dubia* (Southwest Australian isolate).

This procedure is based on the methodology described by USEPA (2002b) for *C.* cf. *dubia* (Method 1002.0), which was adapted for use with the NSW sourced *C.* cf. *dubia* (the Sydney clone) by Bailey et al. (2000). This methodology has been further adapted for use with *C.* cf. *dubia* (Southwest Australian isolate) and Southwest Australian conditions (current study).

In this Southwest Australian test, neonates of *C.* cf. *dubia,* less than 24 hours old, are exposed to the test material at a range of dilutions in a static renewal system until 60% of non-immobilised control organisms have produced at least 15 offspring over 3 broods within 10 days (Figure 14). Test design comprises ten replicates per treatment, each with a single randomly selected neonate. Effects on survival and reproduction are assessed every 24 hours until test termination (maximum of ten days).

This procedure describes routine testing. Test conditions and acceptability criteria are provided in Table 15. Modifications may be made to test requirements to suit region or site-specific conditions within the tolerance ranges presented in Table 6 (Section 2).

3.4.2 Organism culture

Cultures of *C.* cf. *dubia* (Southwest Australian isolate) used for ecotoxicity testing are maintained in glass vessels containing DMW at a temperature of 25 ± 1 °C, pH range 7.8 to 8.3, dissolved oxygen of 90 to 105% saturation (7.4 to 8.6 mg/L) and a conductivity of 180 ± 20 µS/cm. Cultures are maintained in the environmental cabinet with a lighting regime of 16 hour light: 8 hour dark (800 ± 160 Lux).

Health of the culture is critical to the success of tests. The following criteria should be met to ensure healthy test organisms:

- Broodstock mortality < 20% in the 7 day period prior to the use of neonates for testing.
- The first brood of neonates should be produced by the end of Day 5 (but normally by Day 4).
- > 6 neonates should be produced in at least one brood (broodstock must be isolated to determine this). In a beaker of 20 producing females, there should be > 120 neonates.
- Males and ephippeal females should be absent in the broodstock since their presence is considered an indicator of culture stress.
- Cladocerans should show no signs of lethargy or paleness.

Detailed methodology on culture development and maintenance is provided in Appendix D.1 and a Standard operating procedure for culture maintenance for Cladoceran test: *Ceriodaphnia* cf. *dubia* is provided in Appendix D.2.

Source cultures of *C.* cf. *dubia* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Drive, Lane Cove, NSW, Australia, 2066; info@ecotox.com.au.

Table 15: Summary of test conditions and test acceptability criteria for the *Ceriodaphnia* cf. *dubia* (Southwest Australian isolate) partial lifecycle (chronic) toxicity test

Test parameter	Test Condition
Test species	*Ceriodaphnia* cf. *dubia* (Southwest Australian isolate)
Test type	Static renewal
Test duration	Until 60% of non-immobilised control organisms have 3 broods (maximum 10 days)
Temperature	25 ± 1 °C
Light quality	cool-white, fluorescent tube lighting
Light intensity	800 ± 160 Lux
Photoperiod	16 h light : 8 h dark
Test Chamber size	20 mL
Test solution volume	18 mL
Renewal of Test Solutions	24 h or 48 h
Age of test organisms	Less than 24 h old
No. of organisms per replicate	1
No. of replicates per treatment	10
No. of organisms per treatment	10
Feeding regime	*Raphidocelis subcapitata* and freeze dried *Dunaliella salina*, daily
Test chamber aeration	None
Dilution water	Dilute Mineral Water (DMW), prepared by mixing 20% Perrier mineral water with deionised water, with vitamin B12 and selenium supplements
Test concentrations	Minimum of 6 concentrations plus a control
Dilution series	≥ 0.5 dilution series (e.g. 100, 50, 25, 12.5 and 6.25%)
Endpoint	Immobilisation* and reproduction
Reference Toxicant	$CuSO_4.5H_2O$ analytical grade reagent (6.3, 12.5, 25, 50 and 100 µg Cu/L), 5 reps per treatment.
Test acceptability criterion	≥ 80% non-immobilised in controls. An average of ≥ 15 young per non-immobilised female in the controls. 60% of non-immobilised control organisms must produce ≥ 3 broods.

** for the purpose of the test, immobilisation is recorded as an indicator of mortality.*

3.4.3 Equipment

- constant temperature chambers with light cycling
- Pasteur pipettes & bulbs
- pre-prepared Diluent (Dilute Mineral Water, DMW)
- 20 mL borosilicate glass or plastic scintillation vials, depending on the contaminants being tested
- pH, Temperature, Conductivity and Dissolved Oxygen meters (and associated calibration fluids)
- various borosilicate glassware, including 600 mL and 250 mL beakers, 1 L and 250 mL measuring cylinders, 1 and 3 L Scott (screwcap) bottles
- *S. exspinosus* neonates < 24 hours old from Mass Cultures
- *Raphidocelis subcapitata* food stock (available from the CSIRO Australian National Algae Culture Collection https://www.csiro.au/en/about/facilities-collections/collections/anacc/australian-national-algae-supply-service)
- *Dunaliella salina* food stock (available from https://nutrakol.com/)
- autopipettors (10 to 5000 µL) and polyethylene tips.
- racks to hold 20 mL glass scintillation vials
- BPA-free plastic film.

3.4.4 Test preparation

Test diluent and control water

Test diluent and control water is a standard dilute mineral water (DMW): 20% mineral water: 80% deionised water (USEPA 2002a), prepared at least 4 hours prior to test commencement, to ensure dissolved carbon dioxide and pH have reached equilibrium. This is then modified by adding vitamin B12 and selenium supplement. Vitamin B12 supplement stock solution (20 mg/L) is prepared by adding 0.005 g of vitamin B12 to 250 mL of polished (all impurities and contaminants removed) water. The solution is transferred to a 250 mL Schott bottle for storage at 4 °C for up to three months. Selenium stock solution (100 mg/L) is prepared by adding 0.1109 g of Na_2SeO_3 to 500 mL of polished water. This stock is not to be added directly to DMW. Instead, a working selenium supplement stock (4 mg Se/L) is prepared by adding 10 mL of the 100 mg/L selenium stock to 240 mL deionised water. The working selenium stock solution is transferred to a 250 mL Schott bottle for storage at 4 °C for up to three months.

Note: where a toxicity assessment requires a field-collected diluent e.g. upstream of a discharge point, the field-sourced diluent may not be optimal with respect to the health of the test organisms, resulting in test acceptability criteria (Table 15) not being met. As such, test design should include negative control treatments using both modified DMW and field-sourced diluents that can be statistically compared.

Sample preparation: effluents, receiving water, ground waters and leachates

Volume of test material (sample) required per treatment is 2 L. Sample may need to be pre-filtered through a 60 µm mesh (stainless steel for samples where organic contaminants may be present and nylon where metals may be present). Refrigerated sample must be warmed for 2 to 3 hours in an environmental cabinet set at 25 ± 1 °C prior to testing.

Sample pH is measured to establish whether it is within the range for the test (6.0 – 9.0). If pH is outside of this range, a subsample (100 mL for screening test, 500 mL for a full dilution series test) is to be collected for pH adjustment, by the addition of 0.1 M NaOH or HCl as appropriate until pH is 7.0 ± 0.1. Subsequent test design should incorporate pH control treatments run in parallel to determine pH effect on sample toxicity. If the samples require dechlorination, this can be achieved by the addition of sodium thiosulphate to a subsample at a rate of 6.7 mg/L for every 1 mg /L total chlorine in the sample and subsequent test design should incorporate sodium thiosulphate controls.

Samples with high suspended solids concentration may exhibit a reduction in reproduction compared with control treatments due to *C.* cf. *dubia* consuming suspended solids with food, resulting in a relatively poor dietary intake. Suspended solids may also adhere to gill filaments or interfere with feeding processes. As such, visibly turbid samples should be centrifuged to reduce suspended solids concentration and tested in parallel with the uncentrifuged sample.

3.4.5 Daily test procedure

Day 0

Test setup and test solution preparation

- Test datasheets are prepared for each sample tested.
- Pre-rinsed 20 mL scintillation vials are labelled according to datasheet details, with the ten replicates labelled A to J.
- An additional 20 mL scintillation vial is labelled for each treatment to determine physico-chemical parameters.
- Bulk solutions are prepared (Table 16), allowing for daily renewal of test solutions over a ten day period (unless bulk solutions are unlikely to be stable under refrigeration, in which case solutions should be prepared daily).
- 18 mL of each solution is dispensed into pre-labelled 20 mL scintillation vials, one for each replicate in the dilution series, plus the additional vial for physico-chemical parameters.
- Remaining bulk solution is stored in a refrigerator.

Reference toxicant preparation

Reference toxicant (positive control) testing with dissolved copper (0.45 µm filtered fraction) is undertaken each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established criteria (Appendix D3) may highlight issues with test organism health, the test conditions or laboratory technician competence.

Table 16: Suggested volume required for the preparation of bulk test solutions (at day = 0), based on % dilution of a sample

Concentration (%)	Volume 100% sample (mL)	Volume modified DMW (mL)
DMW (Control)	0	2000
6.3	125	1875
12.5	250	1750
25	500	1500
50	1000	1000
100	2000	0

Reference toxicity testing is undertaken with five replicates of each concentration: 0, 6.3, 12.5, 25, 50, 100 µg Cu/L.

- A 0.1 g Cu/L stock solution is prepared by adding 0.3932 g of $CuSO_4.5H_2O$ to 1 L deionised water in a 1 L volumetric flask. Stock solution is transferred to a Schott bottle and stored in the dark at room temperature until required for use.
- Pre-rinsed 20 mL scintillation vials are labelled with the 6 test concentrations, indicating the replicate identity.
- Reference toxicant bulk test solutions (allowing daily renewal for ten days) are prepared in 2 L glass bottles (Table 17) and stored in the refrigerator.
- 18 mL of each solution is dispensed into pre-labelled 20 mL scintillation vials, one vial for each of five replicates in the dilution series.
- Remaining bulk reference toxicant solutions are stored in the refrigerator.

Addition of test organisms, feeding and measurement of physico-chemical parameters

Neonates (< 24 hours) are used for testing and are to be obtained from healthy cultures (refer Appendix D1 for culture development and maintenance).

- While examining under a dissecting microscope, neonates are isolated from healthy mass cultures using a Pasteur pipette and transferred to a 250 mL beaker containing DMW, ensuring that they are released below the surface of the water.
- Again, while examining under a dissecting microscope, using a clean pipette for each treatment and control, one neonate is randomly assigned to each 20 mL test vessel containing 18 mL test solution, ensuring minimal DMW is transferred with each neonate.
- The end of the pipette is cleaned with blotting paper after each addition to prevent cross-contamination.
- Food (*R. subcapitata*) is added to each test vessel to achieve a suspension of 200,000 cells per mL along with *D. salina* at a rate of 12.5 g/L.
- Test vessels are randomly assigned to a rack, covered with BPA-free plastic film and transferred to the environment cabinet.
- The time of the addition of the first animals to the test solutions is noted as t = 0.
- Dissolved oxygen concentration, conductivity and pH are measured in the designated beakers and recorded.

Table 17: Preparation of bulk reference toxicant test solutions in DMW from 0.1 µg Cu/L stock solution

Concentration (µg Cu/L)	Volume of 0.1 µg Cu/L stock (mL)	Volume DMW (mL)
DMW (Control)	0	2000
6.3	0.125	1999.875
12.5	0.25	1999.75
25	0.5	1999.5
50	1	1999
100	2	1998

Days 1 - 10

Test solution renewal, feeding and measurement of physico-chemical parameters

Test solutions are renewed every 1 to 2 days depending on the stability of the test material. Test organisms are fed daily, and test data is recorded daily.

- Fresh test solutions are prepared in 1 L glass beakers and placed in the 25 ºC environmental cabinet for 2 h to ensure a homogeneous temperature between old and new solutions.
- *R. subcapitata* suspension is added to each vessel containing fresh solutions to achieve 200,000 cells per mL. Freeze-dried *D. salina* is added to each vessel at the rate of 12.5 g/L.
- Prior to renewing solutions, daily observations (refer following section) are recorded on the datasheet.
- Test solutions are renewed one at a time by first removing the neonates from old test solutions using a Pasteur pipette.
- The parental organism is then drawn into the Pasteur pipette and placed horizontally on the pipette rack.
- The contents of the beaker are placed into the corresponding vessel for the old test solutions
- The bottom of the original beaker is rinsed using a wash bottle containing deionised water and the rinsate is poured into a waste beaker.
- Test solution is replaced by pouring 18 mL from the fresh 1L beaker for that treatment (concentration) into the 20 mL test vial.
- The parental organism is then returned to the 20 mL test vial, now containing the fresh solution, by gently ejecting below the surface. Any organisms damaged or dropped are discarded and noted on the data sheet.
- Dissolved oxygen concentrations, pH, temperature and conductivity are recorded in the old and new solutions.

Daily observations

Each day the following observations are undertaken and recorded on the datasheet:

- whether parental animal is alive, dead or showing signs of stress
- number of neonates
- number of aborted eggs or embryos
- presence of any males or females carrying ephippia (resting eggs) – refer USEPA (1993) for guidance on distinguishing between parthenogenic females with eggs in the brood pouch, females carrying ephippia, and males.

Test Termination

Tests are terminated when at least 60% of the non-immobilised control organisms have produced at least three broods, either by day 10 or 11. All observations on test organism immobilisation should occur within 2 hours due to the rapid rate of development of *C.* cf. *dubia.*

- The same data are recorded as for the *Daily Observations.*
- Any test organism observed not to have produced young throughout the duration of the test is to be examined under the microscope to determine whether it is male. This is noted on the corresponding datasheet.
- Dissolved oxygen concentrations, pH, temperature and conductivity are recorded.

3.4.6 Test acceptability criteria

The following criteria are to be met for the test to be considered acceptable:

- ≥ 80% non-immobilised control organisms
- Mean number of offspring per non-immobilised female in controls ≥ 15
- 60% of non-immobilised control organisms must produce ≥ 3 broods
- The 96 hour EC50 estimate for the copper reference toxicant (positive control) must fall within previously established lower and upper warning limits.*
- All physico-chemical parameters are to be maintained throughout the test duration. Specifically, a temperature of 25 ± 1 ºC, pH range 7.8 to 8.3, dissolved oxygen concentration of 90 to 105% saturation (7.4 to 8.6 mg/L) and a conductivity of 180 ± 20 µS/cm.

* The IC50 estimate for the copper reference toxicant must fall within two standard deviations of the running mean as depicted on the Control Chart for Cladoceran test: *C.* cf. *dubia* (Appendix D3). If the IC50 estimate for the reference toxicant falls outside of two standard deviations of the running mean, but within three standard deviations, an investigation into the likely cause of departure is to be initiated (e.g. contamination, culture health, culture fatigue or laboratory technician error), which will inform whether the results may be acceptable. However, if the EC50 estimate falls outside of three standard deviations of the running mean, all tests should be repeated. Note: it is expected that testing laboratories will generate their own control chart with their chosen reference toxicant.

3.4.7 Analysis of test data

Any neonates that were either missing or accidentally injured or killed as a result of handling are deducted from the total number of organisms considered for that treatment. Otherwise, any missing animals are considered to be mortalities as a result of exposure to the test solution.

Test endpoints are calculated for the *C.* cf. *dubia* test on survival and reproduction test data. For estimates using statistical approaches assuming normality and homogeneity, data are screened for normality and homogeneity and transformed where necessary. Mean number of live young produced for each adult female is determined for each treatment at either the time of death of the adult or at the end of the experiment. Standard deviation, standard error and per cent coefficient of variation are calculated for each treatment as an indicator of within-treatment variability. Concentration-response relationships are determined and effect concentrations (ECx), generally EC10 and EC50 estimates, i.e. the concentration which results in a 10% or 50% effect in the time prescribed by the test, are calculated with statistical software suitable for the analysis of toxicity data. The No Significant Effect Concentration (NSEC) – the concentration at which the modelled mean response is statistically indistinguishable from the mean control response, may be calculated through methods described by Fisher and Fox (2023) and Fisher et al. (2023). The No Effect concentration (NEC) – the minimum concentration above which an effect is predicted to occur, may be calculated through methods described by Fox et al. (2010).

Resulting chronic effect datasets are suitable for use in the derivation of water quality guideline values (for various levels of aquatic species protection) using the species sensitivity distribution (SSD) approach, the preferred method for guideline derivation for Australia and New Zealand (ANZG 2018; Warne et al. 2025), and ssdtools software (Thorley et al. 2025; Thorley and Schwarz 2018) through its accompanying web-based interface, shinyssdtools software (Dalgarno 2021, 2018; Fox et al. 2023, 2021).

3.5 TEST PROCEDURE: MICROALGAE, *CHLAMYDOMONAS OVIFORMIS*, *TETRADESMUS OBLIQUUS*, *SCENEDESMUS* SP., AND *CHLORELLA* SP. (SOUTHWEST AUSTRALIAN ISOLATES) GROWTH INHIBITION CHRONIC TOXICITY TESTS

This test procedure applies to four microalgal species within the phylum Chlorophyta (green algae). All organisms within this phylum contain chloroplasts responsible for photosynthesis, which provides their distinctive green colour. They are all unicellular algae but have distinct physical and/or behavioural characteristics and life histories.

Chlamydomonas oviformis Pringsheim 1930 (Figure 15a) is a unicellular green alga within the order Chlamydomonadales. It is oval in shape, 10 to 20 µm in length and 8 to 20 µm wide. It has two equal length whip-like flagella that enable motility for feeding and predator avoidance. *C. oviformis* is capable of phototaxis to enable optimal photosynthesis. The *Chlamydomonas* genus is thought to have global distribution in freshwater wetlands, lakes and rivers (Guiry and Guiry 2022).

Tetradesmus obliquus Turpin 2016 (Figure 15b) is a unicellular green alga in order Sphaeropleales. It is elongated (spindle shaped) without spines or flagella and thus is non-motile. It is typically 8 to 13 µm in length and it is often found forming microcolonies. After asexual division, cells can remain attached along the lateral walls. The *Tetradesmus* genus is thought to have global distribution in freshwater wetlands, lakes and rivers (Guiry and Guiry 2022).

Scenedesmus sp. Meyen 1829 (Figure 15c) is a unicellular green alga in the order Sphaeropleales. Cells are elongated and can form colonies of 2, 4, 8, 16 or 32 cells aligned along the lateral walls. Each cell is typically 10 to 20 µm in length and 2 to 3 µm wide. *Scenedesmus* sp. is non-motile and the end cells of a colony typically have two long spines up to 200 µm in length. The *Scenedesmus* genus is thought to have global distribution in freshwater lakes and rivers (Guiry and Guiry 2022).

Chlorella sp. (Figure 15d) is a unicellular green alga within the order Chlorellales. It is spherical in shape, 2 to 10 µm in diameter, without spines or flagella and therefore non-motile. The *Chlorella* genus is thought to have global distribution in freshwater wetlands, lakes and rivers (Guiry and Guiry 2022).

The Southwest Australian isolates were collected from low-flow rivers, East Brook in the Warren River catchment and Yanmah Brook in the Donnelly River catchment.

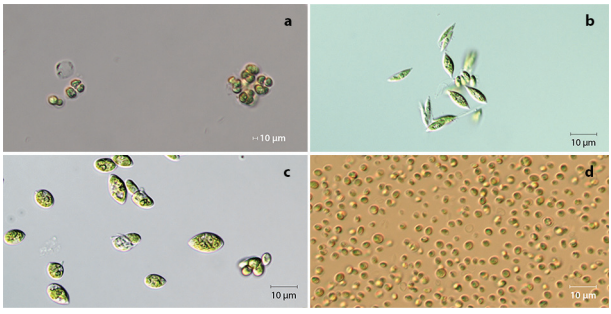


Figure 15: Southwest Australian Toxicity Test Suite microalgal species: a) *Chlamydomonas oviformis*, b) *Tetradesmus obliquus*, c) *Scenedesmus* sp., d) *Chlorella* sp. Photo credit: D. Halliday and M. Larsson

3.5.1 Method summary

This standardised procedure can be used to estimate the chronic toxicity of single chemicals, complex effluents, leachates, receiving waters and elutriates of sediments to the four freshwater micro agal chlorophyte species: *Chlamydomonas oviformis, Tetradesmus obliquus*, *Scenedesmus* sp., *Chlorella* sp. (Southwest Australian isolates).

This procedure is based on the standard method described by USEPA (2002b) (Method 1003.0) modified for use with Southwest Australian species and conditions. The major modification to the pre-existing method is a reduction in test duration from 96 hours to 72 hours, in accordance with Stauber et al. (1995) and the international standard OECD guideline 201 (2006). For these tests, algal populations in logarithmic growth phase are exposed to the test material at a range of dilutions in a static system (Figure 16). Effect on population growth, expressed as cell yield, is assessed. Test design comprises four replicates per treatment (eight replicates per primary control), each with an initial cell density of 10,000 cells/mL. At test cessation, algal cell density is determined indirectly by measuring absorbance. Alternatively, this may be assessed directly using a haemocytometer, particle counter or flow cytometer.

This procedure describes routine testing. Test conditions and acceptability criteria are provided in Table 18. Modifications may be made to test requirements to suit region or site-specific conditions.

3.5.2 Organism culture

Cultures of each microalgal test species (*Chlamydomonas oviformis, Tetradesmus obliquus*, *Scenedesmus* sp., and *Chlorella* sp.) used for ecotoxicity testing are maintained in 100 mL glass flasks at a temperature of 25 ± 1 °C, either on a light table or in an environmental chamber under continuous lighting supplied by cool-white fluorescent or equivalent LED tubes of light intensity 4440 to 8800 Lux.

100 mL liquid cultures should be inoculated from an axenic source of each species (from liquid starter cultures, in-house agar plate cultures or liquid cultures that are re-cultured on a weekly basis). Cultures are inoculated 4 to 7 days prior to testing to ensure the cultures are in their exponential growth phase at test commencement. *C. oviformis* is biflagellate and is therefore motile, thus laboratory cultures do not require agitation to keep cells in suspension. Conversely, *Scenedesmus* sp., *T. obliquus* and *Chlorella* sp. are non-motile species that require manual resuspension by vigorous swirling of flasks containing cultures twice daily by hand, or by culturing on a shaker at a speed of 150 rpm, ensuring that all settled algae are resuspended to maximise their exposure to light and nutrients, to achieve cultures in exponential growth phase.

Culture health is critical to the success of the tests. Healthy cultures in exponential growth phase should appear bright green. Any cultures exhibiting signs of clumping may indicate bacterial contamination and should be discarded.

Detailed methodology on culture development and maintenance is provided in Appendix E.1 and a Standard operating procedure for culture maintenance is provided in Appendix E.2.

Source cultures of *Chlamydomonas oviformis, Tetradesmus obliquus*, *Scenedesmus* sp., and *Chlorella* sp. (Southwest Australian isolates) may be obtained from Ecotox Services Australia, 27/2 Chaplin Drive, Lane Cove, NSW, Australia, 2066; info@ecotox.com.au.

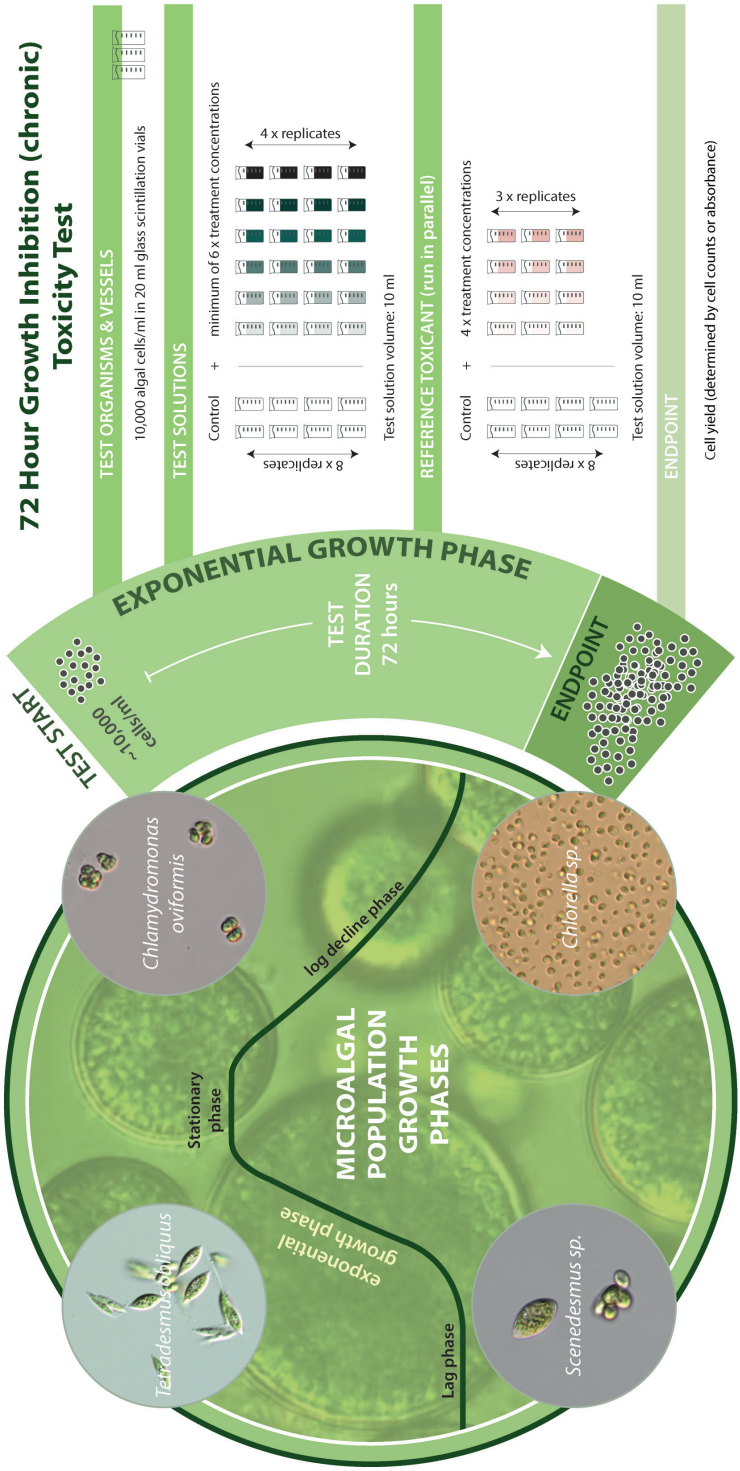


Figure 16: Microalgal lifecycle and toxicity test experimental design details

Table 18: Summary of test conditions and test acceptability criteria for the microalgal growth inhibition (chronic) test for the four microalgal species: *Chlamydomonas oviformis*, *Tetradesmus obliquus, Scenedesmus* sp., *Chlorella* sp.

Test parameter	Test Condition
Test species	*Chlamydomonas oviformis, Tetradesmus obliquus*, *Scenedesmus* sp., *Chlorell*a sp.
Test type	Static, non-renewal
Test duration	72 hours
Temperature	25 ± 1 °C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3 °C during the test
Light quality	cool white fluorescent lighting
Light intensity	4440 to 8800 Lux
Photoperiod	12 h light: 12 h dark
Test Chamber size	20 mL scintillation vial
Test solution volume	10 mL
Renewal of Test Solutions	None
Age of test organisms	Inoculum prepared from 4 to 7 day old stock cultures
Initial density in test vials	10,000 cells/mL
No. of replicates per treatment	4 (8 reps for primary control)
Shaking rate	Twice daily by hand (except *Chlamydomonas oviformis*), which does not require shaking
Aeration	None required
Dilution water	USEPA media
Test concentrations	Minimum of 6 concentrations plus a control
Dilution series	≥ 0.5 dilution series (e.g. 100, 50, 25, 12.5 and 6.25%)
Endpoint	Cell yield, as determined via cell counts or absorbance at 750 nm
Reference Toxicant	$CuSO_4.5H_2O$ analytical grade reagent. Reference toxicant concentration range: 6.3, 12.5, 25 and 50 µg Cu/L for *Scenedesmus* sp., *Tetradesmus obliquus, Chlorella* sp; 6.3, 12.5, 25, 50, 100 µg Cu/L for *Chlamydomonas oviformis*. 3 reps per treatment.
Test acceptability criterion	Mean cell density of at least 160,000 cells per mL in controls. Variability (CV%) in controls should not exceed 20%.

3.5.3 Equipment

- USEPA media (with or without EDTA, Refer Appendix E2, Table E.2.1)
- 20 mL scintillation vials
- multi-parameter digital water quality meter
- 250 mL beakers
- 100 and 200 mL volumetric flasks
- 200 mL measuring cylinders
- *Scenedesmus* sp.*, Tetradesmus obliquus, Chlorella* sp.*, and Chlamydomonas oviformis* cultured in USEPA media
- autopipettes and tips (various sizes)
- light table with between 4440 and 8800 Lux, or constant temperature chamber at 25 ± 1 °C with light (4440 - 8800 Lux) cycling
- spectrophotometer.

3.5.4 Test preparation

Test diluent and control water

The test diluent and control water for the microalgal tests is USEPA media (Appendix E.2, Table E.2.1) prepared daily, at least 4 hours prior to test setup.

Note: where a toxicity assessment requires a receiving water (or reference site water) to be used as a dilution water, it must be filtered (0.45 µm GF/A or GF/C filter) prior to use and nutrient stock solutions must be added to the receiving water prior to preparing the dilutions.

Sample preparation: effluents, receiving water, ground waters and leachates

Volume of test material (sample) required per treatment is 2 L. The sample should either be filtered (0.45 µm GF/A or GF/C filter) or centrifuged to remove indigenous algae. Refrigerated sample must be warmed for 2 to 3 hours in an environmental cabinet set at 25 ± 1 °C prior to testing.

Sample pH is measured to establish whether it is within the range for the test (6.0 – 9.0). If pH is outside of this range, a subsample (100 mL for screening test, 500 mL for a full dilution series test) is to be collected for pH adjustment, by the addition of 0.1 M NaOH or HCl as appropriate until pH is 7.0 ± 0.1. Subsequent test design should incorporate pH control treatments run in parallel to determine pH effect on sample toxicity. De-chlorination (if required) is achieved by the addition of sodium thiosulphate to a subsample at a rate of 6.7 mg/L for every 1 mg/L total chlorine in the sample. Subsequent test design should incorporate parallel sodium thiosulphate control treatments.

Samples with high suspended solids concentration (e.g. some ground waters and receiving waters) may exhibit a reduction in cell yield due to the physical inhibition of light through the test vessels. As such, visibly turbid samples should be centrifuged or filtered to reduce suspended solids concentration and tested in parallel with the uncentrifuged or unfiltered sample.

3.5.5 Daily test procedure

Day 0

Test setup and test solution preparation

- Test datasheets are prepared for each sample tested.
- Pre-rinsed 20 mL scintillation vials are labelled according to datasheet details, with the four (or 8 for controls) replicates labelled A to D (or H).
- If absorbance is used for determining algal growth, an additional 20 mL scintillation vial is labelled for each test treatment (e.g. Control-Blank). This will not be inoculated with algae but used as a blank reading for measuring absorbance at the end of the test.
- 0.2 mL of USEPA media stock solutions #1 to 5 (Appendix E.2, Table E.2.1) are added in order to 200 mL of test sample.
- 250 mL labelled beakers are used to make up each test treatment concentration and controls.
- 10 mL of each test treatment sample is transferred to the corresponding 20 mL scintillation vials using an auto pipette.
- Conductivity and pH are measured in the residual test solutions and controls in the labelled 250 mL glass beakers and recorded on the corresponding datasheets.

Reference toxicant preparation

Reference toxicity testing with dissolved copper (0.45 µm filtered fraction) is to be undertaken each time a toxicity test is performed to assess the sensitivity of the test organisms in addition to laboratory technician competence in performing the tests. Reference toxicity testing is undertaken with three replicates of each concentration. Reference toxicant concentration range is: 0, 6.3, 12.5, 25, 50 µg Cu/L for *Tetradesmus obliquus*, *Scenedesmus* sp. and *Chlorella* sp; and 0, 6.3, 12.5, 25, 50, 100 µg Cu/L for *Chlamydomonas oviformis.*

- A 0.1 g Cu/L stock solution is prepared by adding 0.3932 g of $CuSO_4.5H_2O$ to 1 L deionised water in a 1 L volumetric flask. Stock solution is transferred to a Schott bottle and stored in the dark at room temperature until required for use.
- Pre-rinsed 20 mL scintillation vials are labelled with the five test concentrations, indicating the replicate identity.
- Five 100 mL beakers are labelled with the five test concentrations.
- The reference toxicant test solutions are prepared by serial dilution of the top concentration (50 µg Cu/L for *Scenedesmus* sp., *T. obliquus*, and *Chlorella* sp. or 100 µg Cu/L for *C. oviformis*.
- 10 mL of each reference toxicant test solution is transferred to the corresponding 20 mL scintillation vials using an auto pipette.

Once all test solutions have been prepared, the test vessels are inoculated with the algae according to the following procedure.

Inoculum preparation

Each algal inoculum is to be prepared at a density of 1,000,000 cells/mL through the following process:

- Within three hours prior to the beginning of the tests, algal cells are harvested from a 4 to 7 day old stock culture in exponential growth phase. Algal cells are rinsed to remove nutrient-enriched culture media by centrifuging 20 to 50 mL in a centrifuge tube at 3000 rpm or 1000 g for 5 minutes.
- The supernatant is decanted, and cells are resuspended in 10 mL of USEPA media.
- Centrifugation, decantation and resuspension of the cells is repeated twice. The cell concentrate is mixed to ensure even distribution and cell density is determined using a haemocytometer.
- The cell concentrate is diluted to obtain a cell density of 1,000,000 cells/mL. This is the inoculum.

Test initiation – Inoculation of test solutions

- The inoculum is mixed by swirling and 100 µL is transferred to 10 mL of test solution in each 20 mL test vessel to achieve an initial cell density of 10,000 cells/mL. The test commences at this time.
- If absorbance is used as a measure of cell density at test termination, 10 mL of diluent is added to the remaining inoculum solution and incubated under the same conditions as the test vessels. This vial will be used as a reference to determine the conversion factor between absorbance and cell count at the end of the test.
- Test initiation time is recorded, and each vial is covered with BPA-free plastic film and randomly arranged in an environmental cabinet with continuous light (4440 to 8800 Lux) at a temperature of 25 ± 1 °C.

Days 1 to 3

- Test vessels are gently swirled on a shaker table or by hand twice daily throughout the test to avoid gas limitation, and to encourage photosynthesis, circulation and culture health.

Test Termination

Tests are terminated 72 hours after initiation, whereupon algal growth in each test is determined by measuring absorbance (at 750 nm) as a surrogate for algal biomass using a spectrophotometer. An overview of the spectrophotometer method is provided (following) but it is anticipated that the spectrophotometer-specific protocol should be followed within the testing laboratory. Alternatively, algal growth can be determined by estimating cell density in each test vial using a haemocytometer (e.g. Trenfield et al. 2020) or by flow cytometry (Stauber et al. 2005).

Spectrophotometer method: determination of solution absorbance at 750 nm

- A wavelength of 750 nm is selected on the spectrophotometer.
- The scintillation vials are removed from the environmental cabinet and arranged by treatment.
- The blank for each treatment is analysed first to set as a baseline by transferring to a cuvette for absorbance analysis.
- Each scintillation vial is swirled prior to analysis to ensure all agal cells are in suspension.
- Absorbance of each test solution is recorded and the solutions are transferred back to the corresponding scintillation vials, rinsing the cuvette with polished water in between.
- The spectrophotometer is reset (to zero) in between treatments using the corresponding blank.

- A standard solution with a known absorbance of 750 nm is used to calibrate the spectrophotometer (refer to specific protocol accompanying the standard solution).
- The accuracy of the procedure is assessed from the agreement between the result found and the certified value for the standard solution.

Determination of the conversion factor between absorbance, cell count and yield

- The culture vessel containing the inoculum is retrieved and a subsample of the algal solution is diluted with polished water to achieve an absorbance slightly higher than the higher absorbance recorded for the test solution.
- Cell density is determined using a haemocytometer by counting a minimum of 100 algal cells per grid. This is repeated three times. Then, two dilutions of algal solution are prepared and cell concentrations in the three solutions are recorded based on the density calculated from the initial haemocytometer count. A concentration of zero cells/mL (polished water) is also included to ensure that the series of dilution covers the test measured values.
- Each solution including the polished water is run at 750 nm and the absorbance is recorded.
- This relationship is used to calculate the average cell concentration in the control at 72 hours (test termination).
- Note: the specific protocol accompanying the spectrophotometer should be referred to for detailed methodology.
- Algal cell yield at 72 hours is the endpoint and is calculated as follows:

$$\text{Yield} = \text{cell density}_{t=72\,h} - \text{cell density}_{t=0\,h}$$

3.5.6 Test acceptability criteria

The following criteria are to be met for the test to be considered acceptable:

- Mean algal cell density > 160,000 cells/mL in the controls.
- The % coefficient of variation ≤ 20% in the controls.
- The 72 hour IC50 estimate for the copper reference toxicant (positive control) must fall within previously established lower and upper warning limits*.
- All physico-chemical parameters are to be maintained throughout the test duration. Specifically, temperature at 25 ± 1 °C, pH range of 6 to 9.

* The 72 hour IC50 estimate (the concentration that results in 50% inhibition in cell yield) for the copper reference toxicant must fall within two standard deviations of the running mean as depicted on the *Control Charts for Microalgal tests* (Appendix E.3). If the IC50 estimate for the reference toxicant falls outside of two standard deviations of the running mean, but within three standard deviations, an investigation into the likely cause of departure is to be initiated (e.g. contamination, culture health, culture fatigue or laboratory technician error), which will inform whether the results may be acceptable. However, if the IC50 estimate falls outside of three standard deviations of the running mean, all tests should be repeated. Note: it is expected that testing laboratories will generate their own control chart with their chosen reference toxicant.

3.5.7 Analysis of test data

Test endpoints for each algal test are calculated on cell yield data. For estimates using statistical approaches assuming normality and homogeneity, data are screened for normality and homogeneity and transformed where necessary. Mean cell yield is determined for each treatment at the end of the experiment. Standard deviation, standard error and per cent coefficient of variation are calculated for each treatment as an indicator of within-treatment variability. Concentration-response relationships are determined and effect concentrations, generally IC10 and IC50 estimates, i.e. the concentration which results in a 10% or 50% inhibition in cell yield in the time prescribed by the test, are calculated with statistical software suitable for the analysis of toxicity data. The No Significant Effect Concentration (NSEC) – the concentration at which the modelled mean response is statistically indistinguishable from the mean control response, may be calculated through methods described by Fisher and Fox (2023) and Fisher et al. (2023). The No Effect concentration (NEC) – the minimum concentration above which an effect is predicted to occur, may be calculated through methods described by Fox et al. (2010).

Resulting chronic effect datasets are suitable for use in the derivation of water quality guideline values (for various levels of aquatic species protection) using the species sensitivity distribution (SSD) approach, the preferred method for guideline derivation for Australia and New Zealand (ANZG 2018; Warne et al. 2025), and ssdtools software (Thorley et al. 2025; Thorley and Schwarz 2018) through its accompanying web-based interface, shinyssdtools software (Dalgarno 2021, 2018; Fox et al. 2023, 2021).

4. COMPARATIVE ASSESSMENT OF COPPER TOXICITY

Several toxicity assessments were undertaken with copper sulphate to inform reference toxicant control chart limits for each test in the Southwest Australian test suite (Appendices A.3 to E.3). The comparative sensitivity of the test organisms to copper based on mean 50% effect concentration (EC50) data was *H. viridissima* > *T. obliquus* > *Scenedesmus* sp. > *C. oviformis* > *S. exspinosus* > *C.* cf. *dubia* > *Chlorella* sp. > *G. georgiana* (Table 19).

Table 19: Mean 50% Effect Concentrations (EC50s) for 8 chronic toxicity tests in the Southwest Australian test suite in moderately hard water (70-80 mg $CaCO_3$/L). Species are ordered from most sensitive to least sensitive.

Species	Mean EC50 (µg Cu/L) ± 95% CI
Hydra viridissima	5.4 ± 0.5
Tetradesmus obliquus	25.7 ± 4.2
Scenedesmus sp.	26.4 ± 7.0
Chlamydomonas oviformis	32.6 ± 3.5
Simocephalus exspinosus	33.4 ± 3.0
Ceriodaphnia cf. *dubia*	34.6 ± 3.1
Chlorella sp.	48.9 ± 7.3
Glyptophysa georgiana	127.9 ± 37.7

ACKNOWLEDGEMENTS

This work was funded by the West Australian Government Department of Water and Environmental Regulation (DWER). Collection and preparation of invertebrate and water samples was supported by K. O'Neill, Dr U. Salmon, G. Marino, E. Burns and R.K. Storer (DWER). Culture and method development was conducted by Ecotox Services Australia laboratories, NSW. Chemical analyses were conducted by Envirolab Services Pty Ltd, NSW. Taxonomic identification of *G. georgiana* was confirmed by Dr S. Clarke (Invertebrate Identification); *S. exspinosus* by Dr A. Kotov (Moscow State University); *C.* cf. *dubia* by Dr R. Shiel (University of Adelaide) and algal species by M. Przybylska (Algae Test Consulting) and Dr M. Larsson and D. Halliday (DWER). Analysis of datasets to support site selection was undertaken by Dr U. Salmon, L. Galvin, H. Thornton and maps were prepared by H. Thornton (DWER).

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APPENDICES

APPENDIX A.1: CULTURE DEVELOPMENT, MAINTENANCE AND TEST CONDITION OPTIMISATION FOR THE GASTROPOD MOLLUSC TEST: *GLYPTOPHYSA GEORGIANA* (ENDEMIC TO SOUTHWEST AUSTRALIA)

Adult and juvenile *Glyptophysa georgiana* specimens (50+) were isolated from macrophyte and detritus samples collected from East Brook (Warren River catchment) and Yanmah Brook (Donnelly River catchment) by Department of Water and Environmental Regulation scientists over two sampling periods in December 2020 and February 2021. Upon arrival at Ecotox Services Australia laboratories, isolated snails and egg masses were transferred to 15 L glass aquaria containing 10 L of 0.45 µm filtered bulk water collected from Yanmah Brook. Aquaria were aerated via a mixed bed aquarium filter and snails were fed a single lettuce leaf and five Sera Vipan aquarium food flakes per day. The lettuce leaf was replaced upon signs of deterioration (2-3 days). Adult snails deposited egg masses on the floor and walls of the aquaria.

Once *G. georgiana* cultures were successfully established in the laboratory using bulk water collected from Yanmah Brook, a series of trials was undertaken to determine whether cultures could be maintained in water produced in the Sydney laboratory. Initially, a subset of cultures was maintained in 25% dechlorinated mains water (mains water treated with Sera Aquatan aquarium water conditioning agent, aged for a minimum of 7 days): 75% Yanmah Brook bulk water, and cultures were observed. As no deleterious effect was reported, the proportion of dechlorinated mains water was increased stepwise to 50, 75 and 100% over a six week period. Further trials were undertaken to determine snail responses to food type, electrical conductivity and media type. These trials were conducted on a limited number of snails due to the 16-week developmental time from hatching to maturity and low egg availability during winter months. Results are provided in Tables A.1.1 to A.1.3 respectively.

Food-type trials demonstrated no notable difference in egg production between food types over either 96 hours or 7 days (Table A.1.1). However, some snails were observed to avoid lettuce discs, whereas Sera Vipan food flakes were generally consumed within 24 hours, with no avoidance behaviour observed. As such, Sera Vipan food flakes was selected as the most appropriate food type for future trials and reference toxicant tests.

Table A.1.1: Egg production of *G. georgiana* over 96 hours and 7 days in response to food type*

Food type**	Mean # eggs at 96 h ± SD	Mean # eggs at 7 d ± SD
Sera Vipan lake	14.6 ± 4.0	26.0 ± 4.7
Lettuce disc	13.9 ± 3.8	22.0 ± 3.7

** Trial Parameters: Temperature 25 ± 1°C, Diluent dechlorinated Sydney mains water, Photoperiod 16h light:8h dark, Light intensity: 800±160 Lux, Test Volume 500 mL in 600 mL borosilicate glass squat form beakers, Gentle aeration via a Pasteur pipette. Test Design 10 replicate beakers per treatment, each beaker seeded with a single adult snail. Test solutions renewed on a daily basis. Trial commenced 5 May 2021.*

*** Food type: Sera Vipan treatment - 1 large Sera Vipan flake per surviving snail per day. Lettuce treatment - 1 cut disc of 3 cm diameter per surviving snail per day.*

Table A.1.2: Egg production of *G. georgiana* over 96 hours and 7 days in response to elevated electrical conductivity of the test medium*

Electrical Conductivity (**µS**/cm)**	Mean # eggs at 96 h ± SD	Mean # eggs at 7 d ± SD	% Survival
210	12.4 ± 2.8	22.0 ± 4.2	100
500	12.9 ± 3.4	22.7 ± 4.5	100
1000	12.5 ± 3.9	23.0 ± 2.9	100
2000	12.9 ± 2.6	22.0 ± 3.3	100
3000	13.9 ± 2.9	22.2 ± 4.2	100
6000	2.5 ± 2.5	6.0 ± 6.3	80

** Trial Parameters: Temperature 25 ± 1°C, Diluent dechlorinated Sydney mains water, Photoperiod 16h light:8h dark, Light intensity: 800±160 Lux, Test Volume 500 mL in 600 mL borosilicate glass squat form beakers, Gentle aeration via a Pasteur pipette. Test Design 10 replicate beakers per treatment, each beaker seeded with a single adult snail. Feeding regime: 1 large Sera Vipan flake per surviving snail per day. Test solutions renewed on a daily basis. Trial commenced 22 June 2021.*
*** Electrical Conductivity (EC) test solutions were prepared by adding USEPA GP-2 salts using a magnetic stirrer to Dilute Mineral Water (DMW) with 20% Perrier mineral water (vol/vol.), and measured using calibrated WTW Cond 3110 meter.*

Electrical conductivity trials demonstrated a broad tolerance range from 210 µS/cm to 3000 µS/cm with no notable differences in egg production over either a 96 hour or 7 day exposure period (Table A.1.2). Survival rate was 100% in all treatments except 6000 µS/cm electrical conductivity where survival rate was 80%. As such electrical conductivities of 3000 µS/cm or less are recommended for future trials and reference toxicant tests.

Test media trials demonstrated no notable difference in egg production over either a 96 hour or 7 day exposure period to aged dechlorinated mains water or dilute mineral water (Table A.1.3). As such, aged, dechlorinated mains water was selected for future trials and reference toxicant tests.

Note: adult *G. georgiana* were observed to cease laying eggs during the winter months, despite laboratory conditions remaining constant. Due to a shortage of adult snails to conduct trials during the winter months, test design was altered during the trials to examine test media type such that replicates per treatment were reduced from 10 to 4 with an increase in the number of mature snails in each replicate from 1 to 2. This also reduced the test footprint, thus allowing several assays to be performed simultaneously. This test design was also adopted in the following reference toxicant test program (Appendix A.3).

Since there was no notable difference in rate of egg production between the two time periods (96 hours and 7 days) for each of the parameters tested (food type, media type and electrical conductivity), a 96 hour test duration was selected.

The resulting Standard Operating Procedure for Culture Maintenance is provided in Appendix A.2.

Table A.1.3: Egg production of *G. georgiana* over 96 hours and 7 days in response to test media type*

Media Type**	Mean # eggs at 96 h ± SD	Mean # eggs at 7 d ± SD
Dechlorinated Mains Water	30.3 ± 7.1	55.0 ± 8.2
Dilute Mineral Water	24.5 ± 6.7	54.0 ± 5.9

** Trial Parameters: Temperature 25 ± 1 °C, Photoperiod 16 h light:8 h dark, Light intensity: 800 ± 160 Lux, Test Volume 500 mL in 600 mL borosilicate glass squat form beakers, Gentle aeration via a Pasteur pipette. Test Design 4 replicate beakers per treatment, each beaker seeded with two adult snails. Test solutions renewed daily. Trial commenced 29 September 2021.*
*** Media type: Diluent dechlorinated Sydney mains water using Sera San water conditioner, aged minimum of 1 week. Diluent Dilute Mineral Water (20% Perrier mineral water, vol/vol.).*

APPENDIX A.2: STANDARD OPERATING PROCEDURE FOR CULTURE MAINTENANCE FOR GASTROPOD MOLLUSC TEST: *GLYPTOPHYSA GEORGIANA* (ENDEMIC TO SOUTHWEST AUSTRALIAN)

This standard operating procedure outlines the detailed methodology for maintaining gastropod mollusc, *G. georgiana* snails in mass cultures for use with the standardised toxicity test procedure for Gastropod mollusc, *G. georgiana* (Section 3.1). This procedure is based on that developed by Trenfield et al. (2020) for the Northern Territory snail, *Amerianna cumingi.*

Culture Maintenance Equipment

- constant temperature chambers with light cycling
- glass aquaria, minimum volume 30 L with static or flow through filtration system
- Sera Vipan Tropical Flakes (or equivalent, without dyes or preservatives)
- fresh whole lettuce leaves, rinsed in deionised water
- pre-conditioned and aged mains water
- LDPE vessel to hold pre-conditioned mains water (20 to 100 L)
- water conditioner, such as Sera Aquatan
- pH, temperature, conductivity, and dissolved oxygen meters (and calibration fluids)
- auto-pipettors (10 to 5000 µL) and appropriate disposable polyethylene tips.

Source cultures

The species cultured and referred to as *Glyptophysa georgiana* (Southwest Australian isolate) was originally collected by scientists from the Western Australian Government Department of Water and Environmental Regulation from wild stocks from East Brook (Warren River Catchment). Source cultures of *G. georgiana* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Dr, Lane Cove NSW 2066, ph (02)9420-9480, info@ecotox.com.au.

Preparation of mass culture media

Preconditioned and aged mains water is used to maintain mass cultures (as per method development trials presented in Appendix A.1). However, dilute mineral water (DMW) will be used as a test diluent (Section 3.1). Preconditioned and aged mains water is prepared in 100 L LDPE drums with tap water. LDPE drums must also be aged to avoid leaching of plastic contaminants. Sera Aquatan water conditioner is added (following product instructions) to remove residual chlorine and to neutralise (chelate) any heavy metals that may be present in the water. The pre-conditioned water is vigorously aerated using an aquarium air pump and air stone for one week prior to use. If held under static conditions in glass aquaria, the frequency of water renewals is dependent on the number of snails in culture. A minimum 50% water change is required each week through siphoning and replacing with a transfer pump, ensuring water temperature of the replacement water is similar to that of the aquaria. Snail cultures are fed a combination of Sera Vipan Tropical Flakes and rinsed whole lettuce leaves. Mass culture media is to be maintained at a temperature of 25 ±1 °C, pH of 7.6 to 8.2, dissolved oxygen at 90 to 105% saturation (7.4-8.6 mg/L) and a conductivity of 250 ± 50 µS/cm.

Monitoring culture health

Cultures need to be maintained in good health to ensure an adequate supply of mature egg-laying individuals for testing, and to ensure the quality and consistency of the tests performed. A primary indicator of snail health is egg mass production. Five to 17 eggs will be produced every day or every second day by healthy snails. Eggs will hatch from 4 to 5 days post-laying and most to all eggs are expected to hatch. If snail health is observed to deteriorate, water renewal frequency can be increased and mature snail density can be reduced to 20 individuals per 30 L aquaria. Note: *G. georgiana* has been observed to halt egg production during winter months, even when culture conditions remain consistent over all seasons.

APPENDIX A.3: REFERENCE TOXICANT TESTING AND ESTABLISHMENT OF A CONTROL CHART FOR GASTROPOD MOLLUSC TEST: *GLYPTOPHYSA GEORGIANA* (ENDEMIC TO SOUTHWEST AUSTRALIA)

Reference toxicant (positive control) testing is included each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established values may highlight issues with test organism health, test conditions or performance of test personnel. The EC50 estimate for a reference toxicant is compared to previous EC50 estimates (derived under near identical conditions) using a control chart. The control chart presents the running mean of EC50 estimates ± 2 standard deviations (SD) and ± 3 SD. If the EC50 of a reference toxicant test falls outside of ± 2 SDs but within ± 3 SDs, an investigation of the cause is triggered. Culture conditions, test conditions, verification of reference toxicant test concentrations should all be investigated. If the EC50 for the reference toxicant falls outside of ± 3 SDs of the running mean, the result and all concurrent test results should be rejected.

Control charts ideally consist of 20 individual EC50 estimates, but may be commenced and used with a minimum of five individual EC50 estimates (USEPA 1993). Data used to establish a control chart (reference toxicant: copper sulphate) for *G. georgiana* using the 96 hour egg mass production impairment test are presented in Table A.3.1 and Figure A.3.1. The test concentration range of 31, 63, 125, and 250 µg Cu/L was applied on each testing occasion after a range finding test was undertaken with these concentrations plus 500 and 1000 µg Cu/L. The test data generally represent an un-interrupted dose-response relationship covering no effects to 100% effect and as such are considered suitable for the purpose of a reference toxicant test.

Table A.3.1: The 96 h EC50 estimates for *G. georgiana* egg production for reference toxicant tests with copper sulphate

Test #	96 h EC50 ± SD (µg Cu/L)	95% Confidence Limits
1*	138.0 ± 29.7	6.0-488.9
2	120.4 ± 16.1	65.2-264.4
3	61.5 ± 8.1	18.0-142.5
4	210**	**
5	107.2**	**
6	104.3 ± 9.6	3.0-154.9

** Initial range finding test: nominal test concentrations 31, 63, 125, 250, 500 and 1000 µg Cu/L.*
*** SD and 95% confidence limits not determinable through non-linear interpolation procedure.*

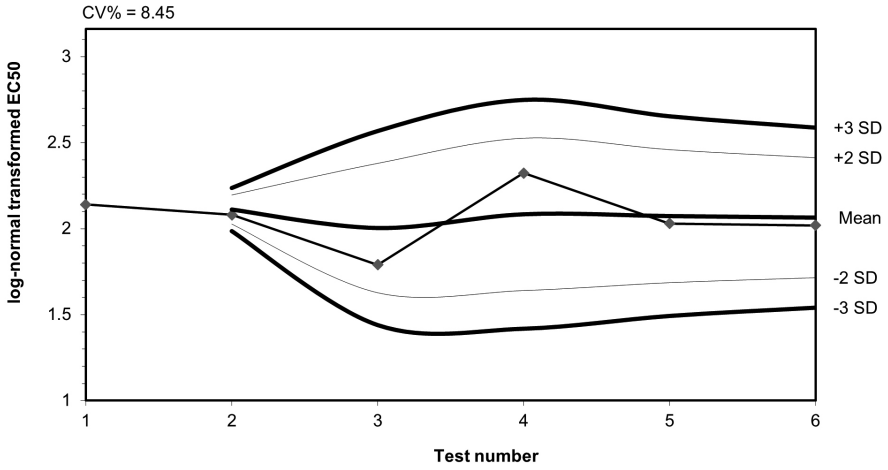


Figure A.3.1: Control chart using log-normal transformed 96 h 50% effect concentration (EC50) estimates (egg production) (µg Cu/L) for reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

APPENDIX B.1 CULTURE DEVELOPMENT, MAINTENANCE AND TEST CONDITION OPTIMISATION FOR CNIDARIAN TEST: *HYDRA VIRIDISSIMA* (SOUTHWEST AUSTRALIAN ISOLATE)

Hydra viridissima specimens were isolated from macrophyte and detritus samples collected from East Brook (Warren River catchment) by Department of Water and Environmental Regulation scientists in December 2020. Bulk water to be used for establishment of cultures was collected from Yanmah Brook and provided ahead of the arrival of samples containing organisms. The bulk water to be used as culture media was filtered to 0.45 µm and refrigerated until required. Repeat collections of bulk water used as culture media were supplied by Department of Water and Environmental Regulation scientists at approximately 4-weekly intervals for a further four months. Cultures were established by isolating a single hydra in a 500 mL glass dish with 300 mL filtered (0.45 µm) bulk water. Hydra were fed daily with twice-rinsed *Artemia salina* nauplii using a Pasteur pipette (according to methods described by Trenfield et al. 2020). Overlying water was replaced 2 to 3 hours after feeding to avoid ammonia build-up in the culture water. Once cultures were established, feeding and frequency of water changes were reduced to three times per week. Cultures were maintained in an environmental chamber with temperature of 25 ± 1 °C, photoperiod 16 h light:8 h dark at 800 ± 160 Lux.

Once *H. viridissima* (Southwest Australian isolate) cultures were successfully established in the laboratory using supplied bulk water, a series of trials were undertaken to determine whether cultures could be maintained in laboratory-produced dilute mineral water (DMW) (20% vol/vol Perrier mineral water and 80% deionised water). The DMW conductivity was raised to 400 µS/cm by the addition of USEPA GP-2 salts (Appendix F). Initially a sub-set of cultures were maintained in a mix of 25% DMW and 75% bulk water without deleterious effect. The proportion of DMW was increased stepwise to 50, 75 and 100% over a six week period. Once cultures were successfully established in DMW, further trials were undertaken to determine hydroid responses to temperature, electrical conductivity, and water hardness to set optimal culture conditions and determine tolerances to some physico-chemical test parameters. Results are provided in Tables B.1.1 to B.1.4.

An initial trial to determine the Specific Growth Rate (SGR), under the same conditions as used in culturing yielded a SGR of 0.23 ± 0.02 hydra/day. This exceeded the test acceptability criterion of 0.17 hydra/day used for the Pilbara isolate of *H. viridissima*, which is routinely used for ecotoxicity testing in Australia (Riethmuller et al. 2003). Trials to assess the tolerance of *H. viridissima* to electrical

Table B.1.1: Growth of *H. viridissima* over 96 h in response to elevated electrical conductivity of the test medium*

Electrical conductivity (**µS**/cm) **	Population size range at 96 h	Mean specific growth rate (± SD)
400	21-28	0.22 ± 0.03
1000	20-29	0.23 ± 0.04
2000	21-25	0.22 ± 0.02
3000	21-28	0.22 ± 0.03
4000	17-23	0.17 ± 0.03
5000	0-5	0.0 ± 0.0

**Trial Parameters: Temperature 25 ± 1 °C, Diluent 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light:8 h dark, Light intensity: 800±160 Lux, Test Volume 30 mL in 50 mL borosilicate glass petri dishes, Test Design 4 replicate dishes per treatment, each dish seeded with ten budding Hydra. Test solutions renewed daily.*

*** Electrical Conductivity (EC) test solutions were prepared by adding USEPA GP-2 salts using a magnetic stirrer to Dilute Mineral Water (DMW) with 20% Perrier mineral water (vol/vol.) and measured using calibrated WTW Cond 3110 meter.*

conductivity demonstrated a broad tolerance range from 120 through to 5000 µS/cm (meeting the test criteria of ≥ 0.17 hydra/day) and minimum mean control population of 20 hydra established for the Pilbara isolate. The Southwest Australian isolate also exceeded these control test criteria for hardness range 80 to 180 mg/L $CaCO_3$. SGR was observed to increase with temperatures in the range 23 to 28 °C, suggesting that the Southwest Australian Isolate may be amenable to culture and testing at 28 °C for tropical applications. In summary the culture and test methodologies used for the Pilbara isolate of *H. viridissima*, adapted from those of a Northern Territory isolate by ERISS (Riethmuller et al. 2003), are suitable for use with the Southwest Australian Isolate.

The resulting Standard Operating Procedure for Culture Maintenance is provided in Appendix B.2.

Table B.1.2: Growth of *H. viridissima* over 96 h in response to lowered electrical conductivity in the test medium. *

Electrical conductivity (**µS**/cm) **	Population size range at 96 h	Mean specific growth rate ± SD
40	0-0	0.0 ± 0.00
80	9-18	0.07 ± 0.06
120	18-26	0.20 ± 0.04
180	24-28	0.24 ± 0.02
250	24-33	0.25 ± 0.04
400	21-26	0.22 ± 0.03

** Trial Parameters: Temperature 25 ± 1 °C, Diluent 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light: 8 h dark, Light intensity: 800±160 Lux, Test Volume 30 mL in 50 mL borosilicate glass petri dishes, Test Design 4 replicate dishes per treatment, each dish seeded with ten budding Hydra. Test solutions renewed daily.*
*** Electrical Conductivity (EC) below 400 µS/cm was achieved by diluting Dilute Mineral Water (DMW) medium of 400 µS/cm with deionised water and measured using calibrated WTW Cond 3110 meter.*

Table B.1.3: Growth of *H. viridissima* over 96 h in response to temperature*

Temperature (°C)	Population size range at 96 h	mean specific growth rate ± SD
23	19-29	0.21 ± 0.05
25	24-30	0.24 ± 0.03
28	29-34	0.29 ± 0.02

** Trial Parameters: Temperature ± 1 °C, Diluent 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume 30 mL in 50 mL borosilicate glass petri dishes, Test Design 4 replicate dishes per treatment, each dish seeded with ten budding Hydra. Test solutions renewed daily.*

Table B.1.4: Growth of *H. viridissima* over 96 h in response to varying DMW media hardness*

Hardness (estimate as mg/L CaCO3)	Mineral water content (% vol/vol)	Population size range at 96 h	Mean specific growth rate ± SD
Very Soft (10-13)	2.5	5-17	0.04 ± 0.06
Soft (40-48)	10	14-24	0.16 ± 0.06
Moderately Hard (80-100)	20	21-29	0.24 ± 0.04
Hard (160-180)	40	19-26	0.21 ± 0.04

** Trial Parameters: Temperature 25 ± 1 °C, Photoperiod 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume 30 mL in 50 mL borosilicate glass petri dishes, Test Design 4 replicate dishes per treatment, each dish seeded with ten budding Hydra. Test solutions renewed daily.*

APPENDIX B.2: STANDARD OPERATING PROCEDURE FOR CULTURE MAINTENANCE FOR CNIDARIAN TEST: *HYDRA VIRIDISSIMA* (SOUTHWEST AUSTRALIAN ISOLATE)

This standard operating procedure outlines the detailed methodology for maintaining cultures for use with the standardised toxicity test procedure for Cnidarian, Hydra viridissima (Southwest Australian isolate) (Section 3.2). It includes methods for maintaining hydra in mass culture, and methods for selecting budding hydroids for use in testing and is based on methodology developed by Riethmuller et al. (2003).

Culture Maintenance Equipment

- constant temperature chambers with light cycling
- glass dishes – 500 mL minimum volume
- 5 L Schott bottle
- BPA-free plastic film
- Perrier mineral water
- deionised water
- magnetic stirrer and Teflon stirring bars
- GP-2 artificial sea salts prepared from AR-grade reagents
- aquarium air pump, 5 mm PVC hose and 1 mL serological pipette
- Pasteur pipette and bulb
- LDPE 20 L water container (clean and aged)
- brine shrimp *Artemia salina* cysts and *Artemia* cyst hatching apparatus
- separation funnel
- 0.5 mL stainless steel sieve (for washing nauplii)
- 100 mL squat form borosilicate glass beakers (for washed *Artemia* nauplii isolates)
- pH, temperature, salinity/conductivity, and dissolved oxygen meters (and associated calibration fluids) auto-pipettors (10 to 5000 µL) and disposable polyethylene tips

Source cultures

The species cultured was originally collected by scientists from the Western Australian Government Department of Water and Environmental Regulation from wild stocks from East Brook (Warren River Catchment). Source cultures of *H. viridissima* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Dr, Lane Cove NSW 2066, ph (02)9420-9480, info@ecotox.com.au.

Preparation of mass culture media

Cultures are maintained in 500 mL glass shallow dishes containing DMW culture medium, modified by raising the conductivity to 800 µS/cm. The frequency of water renewals and feeding is dependent on the testing demands, with a greater frequency of water renewal and feeding required in the lead up to initiating a toxicity test. DMW is prepared at least 4 hours prior to use to allow dissolved carbon

dioxide from the mineral water to be dispelled with aeration allowing pH to reach equilibrium. To prepare 4 L of DMW, 3.2 L of deionised water is mixed with 800 mL Perrier mineral water (i.e. 20% mineral water) in a 5 L Schott bottle. Conductivity is raised to 800 µS/cm by the addition of pre-prepared GP-2 Artificial salts (Appendix F), while stirring with a magnetic stirrer and Teflon stirrer bar. An aeration cap (with serological pipette aeration tip) is fitted to the 5 L Schott bottle and connected to an aquarium aerator ensuring a moderate bubbling rate. Hydra culture media is to be maintained at 25 ± 1 °C, pH 7.8 to 8.3, dissolved oxygen 90 to 105% saturation (7.4-8.6 mg/L) and conductivity 800 ± 80 µS/cm.

Procedure for feeding and renewing culture water

Cultures are maintained in either holding mode or build-up mode. In holding mode, cultures are fed and water is renewed two to three times per week. One week prior to testing, cultures are fed and water is renewed daily to ensure adequate numbers of budding hydra for testing. Feeding is undertaken according to the following procedure (modified from Trenfield et al. 2020).

- Add 1 to 2 g of brine shrimp (*Artemia salina*) cysts to 1 L of filtered seawater in a separation funnel. Incubate this solution in an environmental cabinet at 25 to 30 °C and aerate at a rate that prevents the eggs from settling to the bottom of the funnel. This should occur 1 to 2 days prior to being required to feed the hydra, to allow time for the brine shrimp to hatch from the cysts.
- To harvest the brine shrimp, remove the aeration and allow to stand until cyst casings from hatched cysts have floated to the top of the solution and unhatched cysts gathered at the bottom. Hatched brine shrimp larvae will swim to the bottom of the funnel and will usually be most concentrated just above the unhatched cysts. Place a 100 mL beaker under the funnel and collect the unhatched cysts and dispose of them. Then place a 500 mL beaker under the funnel and collect the next 200 to 500 mL. The brine shrimp will be most concentrated in the first few hundred millilitres of solution.
- The brine shrimp are rinsed prior to being fed to the hydra to remove traces of seawater. The brine shrimp solution is passed through a sieve net and the shrimp caught in the net are transferred into a 100 mL beaker containing polished water. This rinsed solution is passed through the net again and shrimp caught in the net are transferred into another 100 mL beaker containing polished water (Milli-Q® or equivalent). The brine shrimp are now ready to feed to the hydra and should be used as soon as possible.
- Hydra are fed using a Pasteur pipette to gently release the brine shrimp in the vicinity of the hydra so that each hydra can catch at least one brine shrimp in its tentacles.
- Once all hydra have been fed, dishes containing brine shrimp are covered with BPA-free plastic film and placed back into the environmental cabinet with aeration. Feeding time and date are recorded.
- The hydra are left to feed for 2 to 6 hours before water is renewed to avoid build-up of ammonia from uneaten or regurgitated brine shrimp.
- The glass dishes containing the hydra are removed from the environmental cabinet and the water from one of the culture dishes is poured into a sorting tray. Some of the hydra will be poured out of the dish into the sorting tray while some will remain attached to the glass dish.
- Approximately 550 mL of new culture water (modified DMW) is poured into a 600 mL beaker. Approximately 50 mL of this water is poured into the culture dish and swirled to rinse the dish. The rinse water is poured into the sorting tray along with debris and uneaten shrimp from the culture dish. However, if an excessive debris and/or brine shrimp remain, then a second rinse may be required.

- Once the culture dish has been rinsed clean, the remaining 500 mL of new culture water is poured into the culture dish.
- The water in the sorting tray is discarded into a waste container, by holding the tray at an angle so that the water pours out of the tray from a single corner, allowing the hydra to concentrate into that corner and be collected with a Pasteur pipette. As many hydra as possible are transferred back to the culture dish. Alternatively, if the culture is deemed too dirty, a small number of healthy organisms can be transferred to start a new culture.
- The culture is covered with BPA-free plastic film and placed back into the environmental cabinet at 25 ± 1 ºC with gentle aeration.
- Water renewal time is recorded.
- This procedure is repeated for all remaining culture dishes.

Monitoring culture health

Cultures must be maintained in good health to ensure an adequate supply of budding individuals for testing, and to ensure the quality of the tests performed. Healthy hydra cultures will have a high proportion of individuals that have long tentacles, are budding and actively trap brine shrimp when supplied. A decrease in density and/or hydra that are not feeding indicates an unhealthy culture. Hydra within a culture that is unhealthy or too dense may begin to sexually reproduce, in which case, white eggs may be seen protruding from the stalk. Culture water should be replaced, and all glassware should be washed.

APPENDIX B.3: REFERENCE TOXICANT TESTING AND ESTABLISHMENT OF A CONTROL CHART FOR CNIDARIAN TEST: *HYDRA VIRIDISSIMA* (SOUTHWEST AUSTRALIAN ISOLATE)

Reference toxicant (positive control) testing is included each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established values may highlight issues with test organism health, test conditions or performance of test personnel. The EC50 estimate for a reference toxicant is compared to previous EC50 estimates (derived under near identical conditions) using a control chart. The control chart presents the running mean of EC50 estimates ± 2 SD and ± 3 SD. If the EC50 of a reference toxicant test falls outside of ± 2 SDs but within ± 3 SDs, an investigation of the cause is triggered. Culture conditions, test conditions, verification of reference toxicant test concentrations should all be investigated. If the EC50 for the reference toxicant falls outside of ± 3 SDs of the running mean, the result and all concurrent test results should be rejected.

Control charts ideally consist of 20 individual EC50 estimates but may be commenced and used with a minimum of five individual EC50 estimates (USEPA 1993). Data used to establish a control chart (reference toxicant: copper sulphate) for *H. viridissima* (Southwest Australian isolate) using the 96 h population growth test are presented in Tables B.3.1 and Figure B.3.1. The test concentration range of 1.1, 3.3, 10 and 30 µg Cu/L was applied on each testing occasion. The test data generally represent an un-interrupted dose-response relationship covering the range from no effects to zero survival and as such are considered suitable for the purpose of a reference toxicant test.

Table B.3.1: The 96 h EC50 estimates for *H. viridissima* reproduction for reference toxicant tests with copper sulphate

Test #	96 h EC50 ± SD (µg Cu/L)	95% Confidence Limits
1	5.8 ± 0.2	4.5-7.1
2	5.2 ± 0.4	2.2-7.5
3	5.2 ± 0.4	1.8-7.3
4	4.4 ± 0.5	0.9-7.5
5	4.9 ± 0.5	0.6-7.7

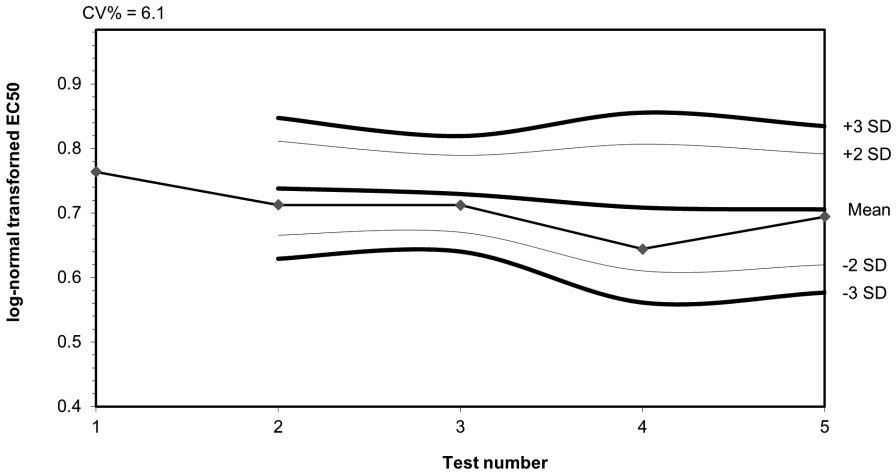


Figure B.3.1: Control chart using log-normal transformed 96 h 50% effect concentration (EC50) estimates (reproduction) (µg Cu/L) for reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

APPENDIX C.1: CULTURE DEVELOPMENT, MAINTENANCE AND TEST CONDITION OPTIMISATION FOR CLADOCERAN TEST: *SIMOCEPHALUS EXSPINOSUS* (SOUTHWEST AUSTRALIAN ISOLATE)

Several cladoceran species were first separated from other taxa in the field by Department of Water and Environmental Regulation (DWER) scientists in December 2020 and March 2021. All cladocerans were transferred into clean food-grade 2 L polyethylene bags containing (250 µm filtered) water from East Brook (Warren River catchment) and Yanmah Brook (Donnelly River catchment) for transport to the laboratory (Section 2.1).

Bulk water (0.45 µm filtered) collected from Yanmah Brook was provided ahead of candidate species samples (for sorting, isolating species and establishing cultures). Repeat collections of bulk water used as culture media were supplied by DWER scientists at approximately 4-weekly intervals for a further four months and refrigerated until required.

In the laboratory, cladocerans were sorted into individual species through the following process. Aliquots of surface water samples containing cladocerans were transferred into 600 mL borosilicate glass beakers. Visibility in the sample was improved by placing the sample in front of a light source and reducing turbidity by adding 400 mL of filtered bulk water to 100 mL of sample. Cladocerans were isolated by eye using a wide bore pipettor and transferred to tissue culture trays with 5 mL wells containing bulk water. Each cladoceran was then examined under a dissecting microscope and categorised into groups based on physical attributes.

Several mass-cultures were established by placing isolates into 600 mL borosilicate glass beakers containing 500 mL of filtered bulk water. All isolated cladocerans were fed using laboratory cultured green alga *Raphicocelis subcapitata* and freeze-dried *Dunaliella* sp. as described in Section 3.3. Initial cultures were maintained for up to 25 days, with cladocerans transferred to fresh beakers containing algal food three times per week. New cultures were established each week for each group of isolates with 40 neonates < 24 h old.

Simocephalus exspinosus individuals were isolated from surface water samples that were collected from East Brook and Yanmah Brook in December 2020 and March 2021. The pH, electrical conductivity, dissolved organic carbon and hardness (as $CaCO_3$) was 7.4, 584 µS/cm, 5.6 mg/L and 74 mg/L, in the East Brook sample; and 7.4, 535 µS/cm, 8.0 mg/L and 73 mg/L in the Yanmah Brook sample.

Once *S. exspinosus* cultures were successfully established in the laboratory using supplied bulk water from Yanmah Brook, trials were initiated to determine if the cultures could be maintained in laboratory-produced dilute mineral water (DMW) (20% vol/vol Perrier mineral water and 80% deionised water). Note: DMW has been used for culturing and toxicity test diluent for several other cladoceran species in Australia (e.g. Bailey et al. 2000). Initially a sub-set of cultures were maintained in a mix of 25% DMW and 75% Yanmah bulk water without deleterious effect. The proportion of DMW was increased stepwise to 50, 75 and 100% over an eight week period.

When *S. exspinosus* cultures were successfully established in DMW, trials were commenced to determine the effects of temperature, electrical conductivity, hardness and feeding rates on longevity and fecundity. Initial trials undertaken in 18 mL DMW in 20 mL scintillation vials were inconclusive due to high variability between individuals with respect to survival, time to first brood and individual brood sizes. A selective breeding program was subsequently undertaken to improve health and consistency of result. This involved isolating neonates from mass cultures using a brood-board format, where a single neonate was placed in a 100 mL borosilicate scintillation vial containing 80 mL of DMW

and fed as described in Appendix C.2. Culture media containing fresh algal food was replaced daily. Between 10 and 20 vials were established on each occasion. Observations of cladoceran condition and number of neonates produced were recorded daily. New mass cultures were established from those surviving individuals that produced at least 12 neonates over a 10 day period. This process was repeated over three occasions to reduce variability in brood sizes and time to release first brood; and to achieve brood sizes and survival rates that were comparable to established criteria for other cultured cladoceran species, such as *C.* cf. *dubia* (Sydney isolate) (Bailey et al. 2000) and used extensively since the early 1990s in Australia for ecotoxicity testing.

Trials were re-commenced with the new cultures and culture vessels to determine optimal feeding regime and tolerance to temperature, electrical conductivity, and water hardness. The results of these trials are presented in Tables C.1.1 - C.1.4.

There appeared to be no benefit to feeding *S. exspinosus* under test conditions at a rate greater than 1x or 1.5x the rate established as optimal for the Sydney clone of another cladoceran, *C.* cf. *dubia* (Bailey et al. 2000). There was a high amount of unconsumed food in the 1, 1.5 and 2x treatments. The 1x rate was subsequently selected for mass culture maintenance and for feeding during chronic toxicity testing.

Table C.1.1: *Simocephalus exspinosus* (Southwest Australian isolate) neonates produced over 10 days in response to different feeding rates of live *Raphicocelis subcapitata* and freeze-dried *Dunaliella salina*. *

Feeding Rate**	Time to first brood (days)	Mean # neonates ± SD
0.5x	5	9.8 ± 4.9
1x	4	11.4 ± 6.8
1.5x	5	12.5 ± 6.9
2x	4	8.1 ± 9.2

** Trial Parameters: Temperature 25 ± 1 °C, Electrical Conductivity 180 µS/cm, Diluent 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light: 8 h dark, Light intensity: 800±160 Lux, Test Volume 80 mL in 100 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed daily.*
*** Feeding rates: 1x was equivalent to feeding rate previously established for another cladoceran (Bailey et al. 2000), refer Section 3.3. The 0.5x treatment was fed 50% of the 1x rate, and the 2x treatment was fed 200% the 1x rate.*

Table C.1.2: *Simocephalus exspinosus* (Southwest Australian isolate) neonates produced over 10 days in response to temperature*

Temperature (°C)	Time to first brood (days)	Mean % survival ± SD	Mean # neonates ± SD
20 ± 1 °C	5	80.0 ± 42.2	8.5 ± 5.6
23 ± 1 °C	4	80.0 ± 42.2	12.1 ± 5.8
25 ± 1 °C	4	80.0 ± 42.2	16.6 ± 6.7
28 ± 1 °C	3	40.0 ±5 1.6	8.0 ± 10.7

** Trial Parameters: Electrical Conductivity 180 µS/cm, Diluent 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light: 8 h dark, Light intensity: 800±160 Lux, Test Volume: Test Volume 80 mL in 100 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed daily.*

On the basis of trial data presented in Tables C.1.2 to C.1.4 several culture and test parameters may be selected such that any chronic test with *S. exspinosus* (Southwest Australian isolate) may be able to achieve those provided in the established test method: *Daphnid Ceriodaphnia dubia, survival and reproduction test Method 1002.0* (USEPA 2002) for a North American cladoceran:

- ≥ 80% non-immobilised control organisms
- Mean number of offspring per non-immobilised female in controls ≥ 15
- 60% of non-immobilised control organisms must produce ≥ 3 broods

Further, with respect to test duration, the test is terminated when 60% or more surviving control females has produced three broods. For the North American isolate of *C. dubia,* this is typically achieved by Day 8. However, for the Southwest Australian isolate, a 9 to 10 day exposure period is typically required to achieve a mean brood size ≥ 15 neonates per surviving female over three broods. In each trial, all surviving females produced at least three broods, meeting the USEPA criterion for *C. dubia*. The parameters relating to control survival, mean brood size and number of broods may be met where the test temperature is 25 ± 1 °C, electrical conductivity is within the range 80 to 2000 µS/cm, hardness is within the range 80 to 180 mg/L as $CaCO_3$, and the test is allowed to run for between nine and ten days.

Table C.1.3: *Simocephalus exspinosus* (Southwest Australian isolate) neonates produced over 10 days in response to electrical conductivity of the test medium *

Electrical Conductivity (µS/cm)**	Time to first brood (days)	Mean % survival ± SD	Mean # neonates ± SD
40	0	0.0 ± 0.0	0.0 ± 0.0
80	4	70.0 ± 48.3	10.0 ± 9.2
180	4	80.0 ± 42.2	16.1 ± 10.1
1000	4	80.0 ± 42.2	15.7 ± 7.7
2000	3	80.0 ± 42.2	16.4 ± 8.8
2500	4	70.0 ± 48.3	15.0 ± 9.5
3000	4	70.0 ± 48.3	16.3 ± 8.7
3500	4	60.0 ± 51.6	11.7 ± 9.4
4000	3	30.1 ± 48.2	7.0 ± 7.7

** Trial Parameters: Temperature 25 ± 1 °C, Diluent 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume: Test Volume 80 mL in 100 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed daily.*
*** Electrical Conductivity below 180 µS/cm was achieved by diluting Dilute Mineral Water (DMW), Electrical Conductivity of 180 µS/cm was achieved using deionised water. Electrical Conductivity treatments above 180 µS/cm were achieved by adding USEPA GP-2 salts (Appendix F) using a magnetic stirrer and calibrated WTW Cond 3110 meter.*

Table C.1.4: *Simocephalus exspinosus* (Southwest Australian isolate) neonates produced over 10 days in response to hardness of the test medium *

Hardness (estimate as mg/L $CaCO_3$)	Mineral water content (% vol/vol)	Mean % survival ± SD	Mean # neonates ± SD
Very Soft (10-13)	2.5	20.0 ± 42.2	0.6 ± 1.9
Soft (40-48)	10	60.0 ± 51.6	10.1 ± 5.7
Moderately Hard (80-100)	20	80.0 ± 42.2	11.4 ± 6.8
Hard (160-180)	40	80.0 ± 42.2	14.4 ± 9.3

** Trial Parameters: Temperature 25 ± 1 °C, Photoperiod 16 h light:8 h dark, Light intensity: 800 ± 160 Lux, Test Volume 80 mL in 100 mL borosilicate glass scintillation vials, Test Design 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed daily.*

APPENDIX C.2: STANDARD OPERATING PROCEDURE FOR CULTURE MAINTENANCE FOR CLADOCERAN TEST: *SIMOCEPHALUS EXSPINOSUS* (SOUTHWEST AUSTRALIAN ISOLATE)

This Standard Operating Procedure outlines the detailed methodology for maintaining cladoceran, *S. exspinosus* (Southwest Australian isolate) in mass cultures and for selecting and isolating neonate cladocerans for use with the standardised toxicity test procedure for cladoceran, *S. exspinosus* (Southwest Australian isolate) (Section 3.3). This procedure is based on that developed by USEPA (1993, 2002), which was validated for use with the NSW sourced 'Sydney clone' of *C. dubia* (Bailey et al. 2000) and has been further adapted for use with *S. exspinosus* (Southwest Australian Isolate) and Southwest Australian conditions (this study). The main point of departure from the USEPA procedures is the change in food source from a combination of digested yeast, Cerophyl and Trout chow to a freeze-dried *Dunaliella salina* ingredient to eliminate risk of bacterial and fungal infections in cultures and associated clumping of algal feed.

Culture Maintenance Equipment

- constant temperature chambers with light cycling
- 600 mL squat form borosilicate glass beakers (for 500 mL mass cultures)
- BPA-free plastic film
- Perrier Mineral Water
- deionised water
- selenium stock solution
- vitamin B12 stock solution
- air pump, 5 mm PVC hose and 1 mL serological pipette
- Pasteur pipette & bulb
- 20 L plastic container, clean and aged to prevent leaching of plastic contaminants
- freeze dried and re-hydrated *Dunaliella salina* supplement- Nutra-Kol (freeze dried *D.salina*)
- 20 mL scintillation vial and racks
- 100 ml squat form borosilicate glass beakers (for neonate isolates)
- *Raphidocelis subcapitata* cultured in Keating's MS Media (Keating 1985)
- pH, temperature, salinity/conductivity, and dissolved oxygen meters (and associated calibration fluids)
- auto-pipettors (10 to 5000 µL) and polyethylene tips

Source cultures

The species cultured was originally collected by scientists from the Western Australian Government Department of Water and Environmental Regulation from wild stocks from East Brook (Warren River catchment) and Yanmah Brook (Donnelly River catchment). Source cultures of *S. exspinosus* (Southwest Australian Isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Dr, Lane Cove NSW 2066, ph (02)9420-9480, info@ecotox.com.au.

Preparation of mass culture media

Cultures are maintained in 500 mL glass beakers (20 to 45 organisms per beaker). Water is renewed on alternate days. At the time of water renewal, cladocerans are fed an algal mixture containing laboratory cultured *Raphidocelis subcapitata* and commercially supplied freeze-dried and re-hydrated *Dunaliella salina* (Nutra-Kol Nutra Red). Cladocerans are reared in a USEPA defined media known as Dilute Mineral Water (DMW), containing trace elements (vitamin B12 and selenium) and food supplements to maintain cladoceran cultures in a healthy condition. The DMW is also used as the diluent and control water for the toxicity tests.

To prepare the vitamin B12 stock solution, 0.005 g vitamin B12 is added to 250 mL polished water (i.e. 20 mg/L) in a 250 mL volumetric flask. This is transferred to a 250 mL Schott bottle and can be stored in a refrigerator at 4 °C for up to three months.

To prepare the selenium stock solution, 0.1109 g of Na_2SeO_3 is added to 500 mL polished water (i.e. 100 mg/L) in a 500 mL volumetric flask. From this, a working stock (4 mg/L selenium) is prepared by adding 10 mL of the 100 mg/L selenium stock to 240 mL deionised water in a 250 mL volumetric flask. This is transferred to a 250 mL Schott bottle and stored in a refrigerator at 4 °C for up to three months.

DMW is prepared at least 4 hours prior to use to allow dissolved carbon dioxide from the mineral water to be dispelled with aeration, allowing pH to reach equilibrium. Four litres of DMW is prepared by mixing 3.2 L of deionised water with 800 mL Perrier mineral water (i.e. 20% mineral water) in a 5 L Schott bottle. Two mL of a 20 mg/L vitamin B12 stock solution is added to 4 L DMW to provide a final concentration of 10 µg/L vitamin B12. 2 mL of a 4 mg/L selenium stock solution is added to 4 L DMW to provide a final concentration of 2 µg/L selenium. The DMW is aerated (moderate bubbling rate) by fitting an aeration cap with a serological pipette aeration tip to the 5 L Schott bottle. DMW is stored at room temperature.

Preparation of Dunaliella salina food supplement stock

Dunaliella salina food supplement stock is prepared by adding 10 mL freeze-dried and re-hydrated *D. salina* to 200 mL polished water in a 250 mL Schott bottle. This can be stored in a refrigerator at 4 °C for up to three months. Final feed rate is 0.25 mL freeze dried *D. salina* per beaker containing 500 mL DMW.

Procedure for mass culture maintenance

Cladoceran culture media is maintained at 25 ± 1 °C, pH 7.8 TO 8.3, dissolved oxygen 90 to 105% saturation (7.4-8.6 mg/L) and conductivity 180 ± 20 µS/cm. 600 mL culture beakers are twice rinsed with deionised water. 500 mL of DMW is transferred to each culture beaker and 0.25 mL freeze dried *D. salina* is added to achieve a final density of 200,000 cells/mL. New mass cultures are initiated by adding 20 to 45 neonates (< 48 hours old) to each culture beaker from other mass cultures or starter cultures, using a Pasteur pipette. Cladocerans are fragile and should be drawn up gradually and released below the water surface. Each culture beaker is covered with BPA-free plastic film and transferred to the 25 °C environment cabinet with a lighting regime of 16 hour light: 8 hour dark (800 ± 160 Lux). Beakers for culture maintenance should never come into contact with chemicals such as detergents and should be washed separately to other glassware.

S. exspinosus, will usually produce their first brood of young on or after Day 4, which usually contains 2 to 4 neonates. Thereafter, broods are usually produced every second day. The second brood usually contains 2 to 6 neonates and the third brood may contain up to 12. Every second day, the number of surviving cladocerans and neonates produced is recorded and cladocerans are transferred to fresh beakers containing fresh DMW and food.

Monitoring culture health

Cultures must be maintained in good health to ensure an adequate supply of healthy individuals for toxicity testing. Cultures may be kept for up to 21 days. The following criteria are to be met:

- Broodstock mortality < 20% in the 7 day period immediately prior to testing.
- The first brood of neonates are to be produced by the end of day 5 (but usually by day 4).
- > 120 neonates per beaker of 20 producing females (> 6 neonates per female) in at least one brood.
- No males or ephippeal females (indicators or culture stress) in cultures.
- Cladocerans show no signs of lethargy and are not pale.

Criteria for selecting neonates for toxicity testing

Neonates to be used for testing are to be held in the culturing cabinet and must be drawn from healthy mass cultures < 24 hours old, from the second brood or greater. They must be healthy, active and uniform in size.

APPENDIX C.3: REFERENCE TOXICANT TESTING AND ESTABLISHMENT OF A CONTROL CHART FOR CLADOCERAN TEST: *SIMOCEPHALUS EXSPINOSUS* (SOUTHWEST AUSTRALIAN ISOLATE)

Reference toxicant (positive control) testing is included each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established values may highlight issues with test organism health, test conditions or performance of test personnel. The EC50 estimate for a reference toxicant is compared to previous EC50 estimates (derived under near identical conditions) using a control chart. The control chart presents the running mean of EC50 estimates ±2 standard deviations (SD) and ±3 SD. If the EC50 of a reference toxicant test falls outside of ±2 SDs but within ±3 SDs, an investigation of the cause is triggered. Culture conditions, test conditions, verification of reference toxicant test concentrations should all be investigated. If the EC50 for the reference toxicant falls outside of ±3 SDs of the running mean, the result and all concurrent test results should be rejected.

Control charts ideally consist of 20 individual EC50 estimates but may be commenced and used with a minimum of five individual EC50 estimates (USEPA 1993). Data used to establish a control chart (reference toxicant: copper sulphate) for *S. exspinosus* (Southwest Australian isolate) using the 3-brood partial life-cycle reproductive impairment test are presented in Table C.3.1 and Figure C.3.1. The test concentration range of 6.3, 12.5, 25, 50 and 100 µg Cu/L was applied on each testing occasion. The test data generally represent an un-interrupted dose-response relationship, covering the range from no effects to zero survival and as such are considered suitable for the purpose of a reference toxicant test.

Table C.3.1: The 10 day EC50 estimates for *S. exspinosus* (Southwest Australian isolate) reproduction for reference toxicant tests with copper sulphate

Test #	10 d EC50 ± SD (µg Cu/L)	95% Confidence Limits
1	37.0 ± 8.4	16.1-47.9
2	26.0 ± 6.4	15.6-44.0
3	29.5 ± 3.5	21.5-37.5
4	36.3 ± 3.5	25.7-43.5
5	36.0 ± 6.7	11.4-48.2

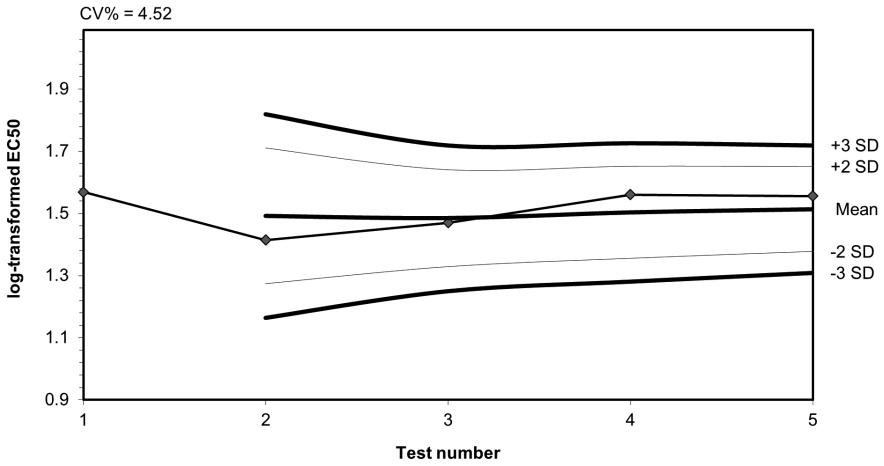


Figure C.3.1: Control chart using log-normal transformed 10 day 50% effect concentration (EC50) estimates (reproduction) (µg Cu/L) for reference toxicant tests with *Simocephalus exspinosus* using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

APPENDIX D.1: CULTURE DEVELOPMENT, MAINTENANCE AND TEST CONDITION OPTIMISATION FOR CLADOCERAN TEST: *CERIODAPHNIA* CF. *DUBIA* (SOUTHWEST AUSTRALIAN ISOLATE)

Several cladoceran species were first separated from other taxa in the field by Department of Water and Environmental Regulation (DWER) scientists in December 2020 and March 2021. All cladocerans were transferred into clean food-grade 2 L polyethylene bags containing (250 µm filtered) water from East Brook (Warren River catchment) and Yanmah Brook (Donnelly River catchment) for transport to the laboratory (Section 2.1).

Bulk water (0.45 µm filtered) collected from Yanmah Brook was provided ahead of candidate species samples (for sorting, isolating species and establishing cultures). Repeat collections of bulk water used as culture media were supplied by DWER scientists at approximately 4-weekly intervals for a further four months and refrigerated until required.

In the laboratory, cladocerans were sorted into individual species through the following process. Aliquots of surface water samples containing cladocerans were transferred into 600 mL borosilicate glass beakers. Visibility in the sample was improved by placing the sample in front of a light source and reducing turbidity by adding 400 mL of filtered bulk water to 100 mL of sample. Cladocerans were isolated by eye using a wide bore pipettor and transferred to tissue culture trays with 5 mL wells containing bulk water. Each cladoceran was then examined under a dissecting microscope and categorised into groups based on physical attributes.

Several mass cultures were established by placing isolates into 600 mL borosilicate glass beakers containing 500 mL of filtered bulk water. Isolated cladocerans were fed using laboratory cultured green alga *Raphicocelis subcapitata* and freeze-dried *Dunaliella* sp. as described in Section 3.4. Initial cultures were maintained for up to 25 days, with cladocerans transferred to fresh beakers containing algal food three times per week. New cultures were established each week for each group of isolates with 40 neonates < 24 h old.

Ceriodaphnia cf. *dubia* individuals were isolated from surface water samples that were collected from East Brook and Yanmah Brook in December 2020 and March 2021. The pH, electrical conductivity, dissolved organic carbon and hardness (as $CaCO_3$) was 7.4, 584 µS/cm, 5.6 mg/L and 74 mg/L, in the East Brook sample; and 7.4, 535 µS/cm, 8.0 mg/L and 73 mg/L in the Yanmah Brook sample.

Once *C.* cf. *dubia* cultures were successfully established in the laboratory using supplied bulk water from Yanmah Brook, trials were initiated to determine if the cultures could be maintained in laboratory-produced dilute mineral water (DMW) (20% vol/vol Perrier mineral water and 80% deionised water). Note: DMW has been used for culturing and toxicity test diluent for several other cladoceran species in Australia (e.g. Bailey et al. 2000). Initially a sub-set of cultures were maintained in a mix of 25% DMW and 75% Yanmah bulk water without deleterious effect. The proportion of DMW was increased stepwise to 50, 75 and 100% over an eight week period.

When *C.* cf. *dubia* (Southwest Australian isolate) cultures were successfully established in DMW, trials were commenced to determine the effects of temperature, electrical conductivity, hardness and feeding rates on longevity and fecundity. Initial trials were inconclusive due to high variability between individuals with respect to survival, time to first brood, and individual brood sizes. A selective breeding program was subsequently undertaken to improve health and consistency of result. This involved isolating neonates from mass cultures using a brood-board format, where a single neonate was placed in a 20 mL borosilicate scintillation vial containing 18 mL of DMW and fed as described in Appendix D.2. Culture media containing fresh algal food was replaced daily. Between 10 and 20

vials were established on each occasion. Observations of cladoceran condition and number of neonates produced were recorded daily. New mass cultures were established from those surviving individuals that produced at least 12 neonates over a 10 day period. This process was repeated over five occasions to reduce variability in brood sizes and time to release first brood; and to achieve brood sizes and survival rates that were comparable to established criteria for other cultured cladoceran species, such as *C.* cf. *dubia* isolated from the Sydney region (Bailey et al. 2000) and used extensively since the early 1990s in Australia for ecotoxicity testing.

Trials were then re-commenced to determine optimal feeding regime and tolerance to temperature, high and low electrical conductivity, and culture water hardness to aid in setting optimal culture conditions and tolerance to some physico-chemical test parameters. The results of these trials are presented in Tables D.1.1 - D.1.4.

Table D.1.1: Mean number of *C.* cf. *dubia* (Southwest Australian isolate) neonates produced over 10 days in response to different feeding rates of live *Raphicocelis subcapitata* and freeze-dried *Dunaliella salina

Feeding rate**	Time to first brood (days)	Mean # neonates ± SD
0.5x	4	12.5 ± 6.5
1x	3	16.4 ±7.9
1.5x	4	17.9 ± 6.5
2x	3	16.4 ± 10.9

**Trial Parameters: Temperature: 25 ± 1 °C, Electrical Conductivity: 180 µS/cm, Diluent: 20% Dilute Mineral Water (Perrier), Photoperiod 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume: 18 mL in 20 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed on a daily basis.*
*** Feeding rates: 1X was equivalent to feeding rate previously established for the Sydney isolate of C. cf. dubia (Bailey et al. 2000) and described in Section 3.4. The 0.5x treatment was fed 50% of the 1x rate, and the 2x treatment was fed 200% the 1x rate.*

There appeared to be no benefit to feeding *C.* cf. *dubia* under test conditions at a rate greater that 1x the rate established as optimal for the Sydney isolate. There was a high amount of unconsumed food in the 1, 1.5 and 2x treatments. The 1x rate was subsequently selected for mass culture maintenance and for feeding during chronic testing.

Table D.1.2: Mean number of *C.* cf. *dubia* (Southwest Australian isolate) neonates produced over 10 days in response to temperature*

Temperature (°C)	Time to first brood (days)	Mean % survival ± SD	Mean # neonates ± SD
20 ± 1 °C	4	80.0 ± 42.2	12.4 ± 6.2
23 ± 1 °C	3	80.0 ± 42.2	17.9 ± 7.1
25 ± 1 °C	3	90.0 ± 31.6	20.6 ± 4.9
28 ± 1 °C	3	60.0 ± 51.6	14.6 ± 12.0

** Trial Parameters: Electrical Conductivity 180 µS/cm, Diluent: 20% Dilute Mineral Water (Perrier), Photoperiod: 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume: 18 mL in 20 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed on a daily basis.*

On the basis of trial data presented in Tables D.1.2 to D.1.4 several culture and test parameters may be selected such that any chronic test with *C.* cf. *dubia* (Southwest Australian isolate) may be able to achieve those provided in Test Method: *Daphnid Ceriodaphnia dubia, survival and reproduction test Method 1002.0* (USEPA 2002) for a North American isolate:

- ≥ 80% non-immobilised control organisms
- Mean number of offspring per non-immobilised female in controls ≥ 15
- 60% of non-immobilised control organisms must produce ≥ 3 broods

Further, with respect to test duration, the test is terminated when 60% or more surviving control females has produced three broods. For the North American isolate, this should be achieved by Day 8. This measure is also used for chronic toxicity tests with the Sydney isolate. However, between eight and ten days are required for the Southwest Australian isolate to achieve this parameter. The parameters relating to control survival, mean brood size and number of broods may be met where the test temperature is 25 ± 1 ºC, Electrical Conductivity is within the range 80 to 3500 µS/cm, hardness is within the range 80 to 180 mg/L as CaCO3, and the test is allowed to run for between eight and ten days.

Table D.1.3: The mean number *C.* cf. *dubia* (Southwest Australian isolate) neonates produced over 10 days in response to electrical conductivity of the test medium*

Electrical conductivity (µS/cm)**	Time to first brood (days)	Mean % survival ± SD	Mean # neonates ± SD
40	0	0.0 ± 0.0	0.0 ± 0.0
80	4	80.0 ± 42.2	9.5 ± 7.0
180	3	100.0 ± 0.0	17.9 ± 2.9
1000	4	90.0 ± 31.6	16.0 ± 6.5
2000	3	80.0 ± 42.2	19.1 ± 5.4
2500	4	90.0 ± 31.6	16.9 ± 5.6
3000	4	80.0 ± 42.2	14.0 ± 6.8
3500	4	90.0 ± 31.6	12.8 ± 5.4
4000	3	70.0 ± 48.3	7.3 ± 3.8

** Trial Parameters: Temperature: 25 ± 1 °C, Diluent: 20% Dilute Mineral Water (Perrier), Photoperiod: 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume: 18 mL in 20 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed on a daily basis. Trial commenced 24 September 2021.*
*** Electrical Conductivity below 180 µS/cm was achieved by diluting Dilute Mineral Water (DMW), Electrical Conductivity of 180 µS/cm was achieved using deionised water. Electrical Conductivity treatments above 180 µS/cm were achieved by adding USEPA GP-2 salts (Appendix F) using a magnetic stirrer and calibrated WTW Cond 3110 meter.*

Table D.1.4: The mean number *C.* cf. *dubia* (Southwest Australian isolate) neonates produced over 10 days in response to hardness of the test medium*

Hardness (estimate as mg/L $CaCO_3$)	Mineral water content (% vol/vol)	Mean % survival + SD	Mean # neonates + SD
Very Soft (10-13)	2.5	10.0 ± 31.6	1.5 ± 4.7
Soft (40-48)	10	70.0 ± 48.3	15.3 ± 7.2
Moderately Hard (80-100)	20	90.0 ± 31.6	20.6 ± 4.9
Hard (160-180)	40	80.0 ± 42.2	17.8 ± 3.7

** Trial Parameters: Temperature: 25 ± 1 °C, Photoperiod: 16 h light: 8 h dark, Light intensity: 800 ± 160 Lux, Test Volume: 18 mL in 20 mL borosilicate glass scintillation vials, Test Design: 10 replicate vials per treatment, each vial seeded with a single neonate < 24 h old. Test solutions renewed on a daily basis.*

APPENDIX D.2: STANDARD OPERATING PROCEDURE FOR CULTURE MAINTENANCE FOR CLADOCERAN TEST: *CERIODAPHNIA* CF. *DUBIA* (SOUTHWEST AUSTRALIAN ISOLATE)

This standard operating procedure outlines the detailed methodology for maintaining cladoceran, *C.* cf. *dubia* in mass cultures and for selecting and isolating neonate cladocerans for use with the standardised toxicity test procedure for Cladoceran: *C.* cf. *dubia* (Southwest Australian isolate) (Section 3.4). This procedure is based on the USEPA (1993 & 2002) method, which was validated for use with the NSW sourced 'Sydney clone' of *C.* cf. *dubia* (Bailey et al. 2000) and has been further adapted for use with *C.* cf. *dubia* (Southwest Australian isolate) and Southwest Australian conditions (this study). The main point of departure from the USEPA procedures is the change in food source from a combination of digested yeast, Cerophyl and Trout chow to a freeze-dried *Dunaliella salina* ingredient to eliminate risk of bacterial and fungal infections in cultures and associated clumping of algal feed.

Culture maintenance equipment

- constant temperature chambers with light cycling
- 600 mL squat form borosilicate glass beakers (for 500 mL mass cultures)
- BPA-free plastic film
- Perrier Mineral Water
- deionised water
- selenium stock solution
- vitamin B12 stock solution
- aquarium air pump, 5 mm PVC hose and 1 mL serological pipette
- Pasteur pipette and bulb
- 20 L plastic container (clean and aged to prevent contamination from leachates)
- freeze dried and re-hydrated *Dunaliella* supplement- Nutra-Kol (freeze dried *Dunaliella*)
- 20 mL scintillation vial and racks
- 100 ml squat form borosilicate glass beakers (for neonate isolates)
- *Raphidocelis subcapitata* cultured in Keating's MS Media (Keating 1985)
- pH, temperature, salinity/conductivity and dissolved oxygen meters (and associated calibration fluids)
- auto-pipettors (10 to 5000 µL) and polyethylene tips

Source cultures

The species cultured was originally collected by scientists from the Western Australian Government Department of Water and Environmental Regulation from wild stocks from East Brook (Warren River catchment) and Yanmah Brook (Donnelly River catchment). Source cultures of *C.* cf. *dubia* (Southwest Australian isolate) may be obtained from Ecotox Services Australia, 27/2 Chaplin Dr, Lane Cove NSW 2066, ph (02)9420-9480, info@ecotox.com.au.

Preparation of mass culture media

Cultures are maintained in 500 mL glass beakers (20 to 45 organisms per beaker). Water is renewed on alternate days. At the time of water renewal, cladocerans are fed an algal mixture containing laboratory cultured *Raphidocelis subcapitata* and commercially supplied freeze-dried and re-hydrated *Dunaliella salina* (Nutra-Kol Nutra Red). Cladocerans are reared in a USEPA defined media known as Dilute Mineral Water (DMW), containing trace elements (vitamin B12 and selenium) and food supplements to maintain cladoceran cultures in a healthy condition. The DMW is also used as the diluent and control water for the toxicity tests.

To prepare the vitamin B12 stock solution, 0.005 g vitamin B12 is added to 250 mL polished water (i.e. 20 mg/L) in a 250 mL volumetric flask. This is transferred to a 250 mL Schott bottle and can be stored in a refrigerator at 4 °C for up to three months.

To prepare the selenium stock solution, 0.1109 g of Na_2SeO_3 is added to 500 mL polished water (i.e. 100 mg/L) in a 500 mL volumetric flask. From this, a working stock (4 mg/L selenium) is prepared by adding 10 mL of the 100 mg/L selenium stock to 240 mL deionised water in a 250 mL volumetric flask. This is transferred to a 250 mL Schott bottle and stored in a refrigerator at 4 °C for up to three months.

DMW is prepared at least 4 hours prior to use to allow dissolved carbon dioxide from the mineral water to be dispelled with aeration allowing pH to reach equilibrium. To prepare 4 L of DMW, mix 3.2 L of deionised water with 800 mL Perrier mineral water (i.e. 20% mineral water) in a 5 L Schott bottle. Two mL of a 20 mg/L vitamin B12 stock solution is added to 4 L DMW to provide a final concentration of 10 µg/L vitamin B12. 2 mL of a 4 mg/L selenium stock solution is added to 4 L DMW to provide a final concentration of 2 µg/L selenium. The DMW is aerated (moderate bubbling rate) by fitting an aeration cap with a serological pipette aeration tip to the 5 L Schott bottle. DMW is stored at room temperature.

Preparation of Dunaliella salina food supplement stock

Dunaliella salina food supplement stock is prepared by adding 10 mL freeze-dried and re-hydrated *D. salina* to 200 mL polished water in a 250 mL Schott bottle. This can be stored in a refrigerator at 4 °C for up to three months. Final feed rate is 0.25 mL freeze dried *D. salina* per beaker containing 500 mL DMW.

Procedure for mass culture maintenance

Cladoceran culture media is maintained at 25 ± 1 °C, pH 7.8 to 8.3, dissolved oxygen at 90 to 105% saturation (7.4-8.6 mg/L) and a conductivity of 180 ± 20 µS/cm. 600 mL culture beakers are twice rinsed with deionised water. 500 mL of DMW is transferred to each culture beaker and 0.25 mL freeze dried *D. salina* is added to achieve a final density of 200,000 cells/mL. New mass cultures are initiated by adding 20 to 45 neonates (< 48 hours old) to each culture beaker from other mass cultures or starter cultures, using a Pasteur pipette. Cladocerans are fragile and should be drawn up gradually and released below the water surface. Each culture beaker is covered with BPA-free plastic film and transferred to the 25 °C environment cabinet with a lighting regime of 16 hour light: 8 hour dark (800 ± 160 Lux). Beakers for culture maintenance should never come into contact with chemicals such as detergents and should be washed separately to other glassware.

C. cf. *dubia* will usually produce their first brood of young on or after Day 4, which usually contains 2 to 4 neonates. Thereafter, broods are usually produced every second day. The second brood usually contains 2 to 6 neonates and the third brood may contain as many as 12. Every second day, the number of surviving cladocerans and neonates produced is recorded and cladocerans are transferred to fresh beakers containing fresh DMW and food.

Monitoring culture health

Cultures must be maintained in good health to ensure an adequate supply of healthy individuals for toxicity testing. Cultures may be kept for up to 21 days. The following criteria are to be met:

- Broodstock mortality < 20% in the 7 day period immediately prior to testing
- The first brood of neonates are to be produced by the end of day 5 (but usually by day 4)
- > 120 neonates per beaker of 20 producing females (> 6 neonates per female) in at least one brood.
- No males or ephippeal females (indicators or culture stress) in cultures.
- Cladocerans show no signs of lethargy and are not pale.

Criteria for selecting neonates for toxicity testing

Neonates to be used for testing are to be held in the culturing cabinet and must be drawn from healthy mass cultures, < 24 h old, from the second brood or greater, healthy, active and uniform in size.

APPENDIX D.3: REFERENCE TOXICANT TESTING AND ESTABLISHMENT OF A CONTROL CHART FOR CLADOCERAN TEST: *CERIODAPHNIA* CF. *DUBIA* (SOUTHWEST AUSTRALIAN ISOLATE)

Reference toxicant (positive control) testing is included each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established values may highlight issues with test organism health, test conditions or performance of test personnel. The EC50 estimate for a reference toxicant is compared to previous EC50 estimates (derived under near identical conditions) using a control chart. The control chart presents the running mean of EC50 estimates ± 2 standard deviations (SD) and ± 3 SD. If the EC50 of a reference toxicant test falls outside of ± 2 SDs but within ± 3 SDs, an investigation of the cause is triggered. Culture conditions, test conditions, verification of reference toxicant test concentrations should all be investigated. If the EC50 for the reference toxicant falls outside of ± 3 SDs of the running mean, the result and all concurrent test results should be rejected.

Control charts ideally consist of 20 individual EC50 estimates but may be commenced and used with a minimum of five individual EC50 estimates (USEPA 1993). Data used to establish a control chart (reference toxicant: copper sulphate) for *C.* cf. *dubia* (WA Isolate) using the 3-brood partial life-cycle reproductive impairment test are presented in Table D.3.1 and Figure D.3.1. The test concentration range of 6.3, 12.5, 25, 50 and 100 µg Cu/L was applied on each testing occasion. The test data generally represent an un-interrupted dose-response relationship, covering the range from no effects to zero survival and as such are considered suitable for the purpose of a reference toxicant test.

Table D.3.1: The 10 day EC50 estimates for *C.* cf. *dubia* (Southwest Australian isolate) reproduction for reference toxicant tests with copper sulphate

Test #	10 d EC50 ± SD µg Cu/L	95% Confidence Limits
1	33.2 ± 2.1	26.3-37.7
2	29.7 ± 6.8	10.4-41.3
3	29.3 ± 5.7	13.2-38.8
4	39.8 ± 1.6	34.3-42.4
5	36.1 ± 3.3	25.4-41.9

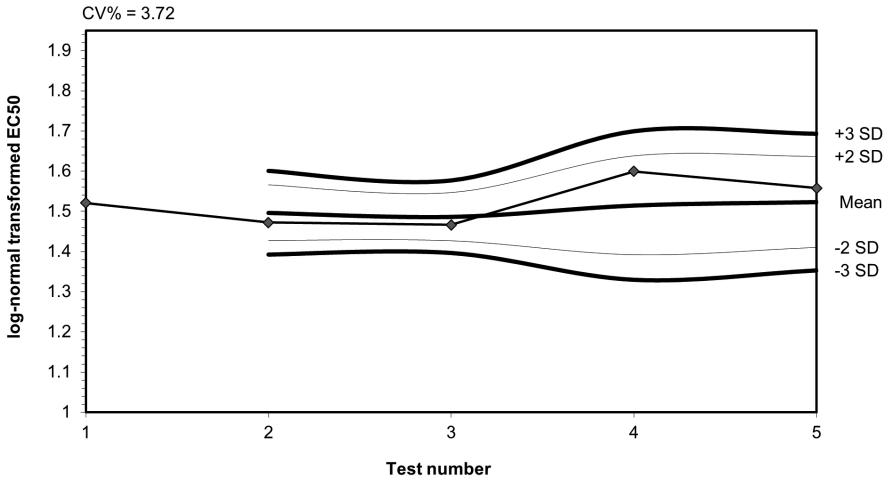


Figure D.3.1: Control chart using log-normal transformed 10 day 50% effect concentration (EC50) estimates (reproduction) (µg Cu/L) for *Ceriodaphnia* cf. *dubia* reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

APPENDIX E.1: CULTURE DEVELOPMENT, MAINTENANCE AND TEST CONDITION OPTIMISATION FOR FOUR CHLOROPHYTE TESTS: *TETRADESMUS OBLIQUUS, CHLAMYDOMONAS OVIFORMIS, SCENEDESMUS* SP. AND *CHLORELLA* SP. (SOUTHWEST AUSTRALIAN ISOLATES)

Microalgal cultures were isolated from surface water samples collected from East Brook (Warren River catchment) and Yanmah Brook (Donnelly River catchment) by Department of Water and Environmental Regulation (DWER) scientists, with the aim of establishing as many axenic single-species cultures as possible. The approach described here is primarily based on the culturing and testing methods for the green alga, *Raphidocelis subcapitata* (formerly *Selenastrum capricornutum*) (USEPA 2002), which uses USEPA media for culturing and ecotoxicity testing. The isolation procedure followed several stages over a ten month period.

The initial phase of the isolation procedure involved drawing 10 mL sub-samples of supplied East Brook and Yanmah Brook bulk waters to inoculate numerous 200 mL sterile USEPA media in 250 mL flasks.

The pH, electrical conductivity, dissolved organic carbon and hardness (as $CaCO_3$) of the East Brook sample was 7.4, 584 µS/cm, 5.6 mg/L and 74 mg/L, respectively. The same analytes from the Yanmah Brook sample were 7.4, 535 µS/cm, 8.0 mg/L and 73 mg/L, respectively. All media became visibly green in colour within 72 hours with a mixed culture of microalgal species. Fresh mixed species cultures were prepared weekly to maintain microalgae in log growth phase by inoculating fresh sterile USEPA media with 10 mL of one week old cultures. This process was repeated over a ten month period once concurrent isolation work was completed.

Isolation of individual species involved a repeated process of plating out mixed-species cultures onto agar plates, isolating individual colonies and breaking these up using glass beads in Macartney bottles, re-plating onto agar plates, repeating this process several times. Sterile agar plates with USEPA media were prepared at least one day prior to use using an autoclave. Inoculations were all performed in a laminar flow cabinet, with the workspace sterilised by UV light for 15 minutes prior to use. Agar plates were inoculated from mixed species liquid cultures with a flamed loop using streak dilution following aseptic techniques. Agar plates were incubated under the same conditions as used for the liquid cultures. Green algal colonies became visible on the agar plate within 2 to 3 weeks of inoculation. Separated individual green colonies were transferred to Macartney bottles containing sterile USEPA media and glass beads using a flamed loop. The contents of the Macartney bottle were vigorously shaken in order to break up the colony into individual cells. A flamed loop was then used to inoculate fresh agar plates, again using a streak dilution technique. Microscopic examination was used to confirm whether a single species was achieved, and if confirmed, liquid cultures were prepared using inoculum from the Macartney bottle solutions. This process was repeated over a six month period, resulting in the isolation of four separate species. Cultures were confirmed as single-species and preliminary taxonomic identification (based on morphology) provided by Ms Malgorzata Przybylska of AlgaeTest Consulting using isolates preserved in Lugol's iodine solution. Taxonomic identification was further assessed and clarified by Dr Michaela Larsson, Department of Water and Environmental Regulation using phylogenetic and morphological assessments, confirming the single-species status of the cultures. These were: *Tetradesmus obliquus, Chlamydomonas oviformis, Scenedesmus* sp. and *Chlorella* sp.

The use of these techniques will likely have been selective for species amenable to culture in USEPA media and to growing on agar media. Several species, including diatoms, observed in liquid mixed cultures were not visible as agar plate colonies. USEPA media was supplemented with sodium silicate to promote diatom growth. However, no diatoms were successfully reared on agar media.

T. obliquus, C. oviformis, Chlorella sp. and *Scenedesmus* sp. are all unicellular green algae (Chlorophyceae) found globally in freshwater lakes, rivers and wetlands. *C. oviformis* Pringsheim 1930 is biflagellate and therefore motile. As such, laboratory cultures do not require agitation to keep cells in suspension. *Scenedesmus* sp., *T. obliquus, Chlorella* sp. are non-motile and require manual resuspension (usually shaking cultures twice daily by hand or by culturing on a shaker table) to maintain cultures in logarithmic growth.

Culture and test optimisation

Once single-species axenic cultures were successfully established, trials were commenced to determine responses to temperature and electrical conductivity to inform optimal culture conditions and also to determine tolerances to some physico-chemical test parameters. The results of these trials are presented in Tables E.1.1 and E.1.2. Trials were also commenced to examine effects of test media pH; however, the relatively poor buffering capacity of USEPA media resulted in pH drifting back to original media pH within hours of adjustment. This trial was abandoned and the results not presented here.

Table E.1.1: Microalgal growth (cell yield) over 72 hours in response to temperature*

Temperature (°C)	Cell Yield (Mean number of cells/mL x10^4 ± SD)			
	T. obliquus	*C. ovifomis*	*Scenedesmus* **sp.**	*Chlorella* **sp.**
21 ± 1 °C	24.6 ± 1.8	24.1 ± 1.0	26.8 ± 2.1	20.1 ± 1.1
23 ± 1 °C	26.0 ± 3.1	25.0 ± 1.4	29.6 ± 2.0	21.2 ± 1.9
25 ± 1 °C	25.5 ± 1.4	26.2 ± 1.0	33.1 ± 2.5	22.3 ± 1.8
28 ± 1 °C	27.1 ± 1.9	27.4 ± 1.8	27.1 ± 4.4	25.0 ± 1.2

** Trial Parameters: Diluent USEPA media, Photoperiod 12 h light:12 h dark, Light intensity: 4440-8800 Lux, test volume 10 mL in 20 mL borosilicate glass scintillation vials, manually re-suspended twice per day. Test design: 4 replicate vials per treatment, each vial seeded with 10,000 cells/mL.*

Table E.1.2: Microalgal growth (cell yield) over 72 hours in response to elevated electrical conductivity of the test medium.*

Electrical Conductivity (**µS**/cm)**	Cell Yield (Mean number of cells/mL x10^4 ± SD)			
	T. obliquus	*C. ovifomis*	*Scenedesmus* sp.	*Chlorella* sp.
100	26.6 ± 1.7	25.1 ± 2.2	28.1 ± 1.1	21.6 ± 1.5
500	26.3 ± 1.7	26.3 ± 1.7	27.5 ± 1.1	22.4 ± 2.1
1000	25.8 ± 2.2	26.3 ± 1.2	26.4 ± 1.6	23.4 ± 1.6
2000	24.6 ± 1.3	25.7 ± 1.9	24.7 ± 1.8	21.4 ± 0.8
3000	21.6 ± 2.0	23.4 ± 3.5	22.7 ± 2.1	20.4 ± 1.5
4000	17.0 ± 2.4	16.5 ± 3.8	8.6 ± 5.0	8.6 ± 5.9

** Trial Parameters: Temperature 25 ± 1 °C, Diluent USEPA media, Photoperiod 12 h light:12 h dark, Light intensity: 4440-8800 Lux, test volume 10 mL in 20 mL borosilicate glass scintillation vials, manually re-suspended twice per day. Test design: 4 replicate vials per treatment, each vial seeded with 10,000 cells/mL.*

*** Electrical conductivity (EC) test solutions were prepared by adding USEPA GP-2 salts using a magnetic stirrer to USEPA media and measured using calibrated WTW Cond 3110 meter.*

One of the test acceptability criteria for the USEPA methodology with *R. subcapitata* is that the mean cell yield must be equal to or greater than $16.0x10^4$ cells/mL. With respect to test temperature, this criterion is readily met with the four Southwest Australian isolated species at test temperatures in the range 21 to 28 °C (Table E.1.1). The lowest cell yield occurred at 21 °C for all species tested. While growth was highest at 28 °C for *Chlorella* sp.*, T. obliquus* and *C. oviformis*, all cultures performed well at 25 °C, which is a more representative test temperature for Southwest temperate systems. All four WA microalgal isolates exceeded the ≥ $16.0x10^4$ cells/mL test criterion over the electrical conductivity range of between 100 and 3000 µS/cm (Table E.1.2). Electrical conductivity over 3000 µS/cm may inhibit algal growth and should be taken into consideration should any of these species be used to assess samples that exceed this threshold.

APPENDIX E.2: STANDARD OPERATING PROCEDURE FOR CULTURE MAINTENANCE FOR FOUR CHLOROPHYTE TESTS: *TETRADESMUS OBLIQUUS, CHLAMYDOMONAS OVIFORMIS, SCENEDESMUS* SP. AND *CHLORELLA* SP. (SOUTHWEST AUSTRALIAN ISOLATES)

This standard operating procedure outlines the detailed methodology for maintaining liquid cultures of *Tetradesmus obliquus, Chlamydomonas oviformis, Scenedesmus* sp. *and Chlorella* sp. (Southwest Australian isolates) in mass cultures for use with the Standardised Toxicity Test Procedures for Microalgae: *Tetradesmus obliquus, Chlamydomonas oviformis* and *Scenedesmus* sp. *and Chlorella* sp. (Southwest Australian isolates) (Section 3.4.). This procedure is based on that developed by USEPA for *R. subcapitata* (USEPA 2002) and APHA for *Chlorella vulgaris* (APHA 1998).

Culture maintenance equipment

- 250 mL sterilised conical flasks
- 250 or 500 mL Schott Bottles
- autoclave, autoclave tape, autoclave bags, non-absorbent cotton wool, aluminium foil
- EPA Media Sock Solutions and associated analytical grade reagents
- deionised water
- Bunsen burner or propane burner
- laminar flow cabinet with UV lamp
- light table (4440 - 8800 Lux) or constant temperature chamber at 25 ± 2 °C with light (4440 - 8800 Lux) cycling
- microscope, haemocytometer, slides and cover slips
- Buchner funnel and filtration apparatus, 0.2 µm nitrocellulose acetate filter membrane
- 1 to 2 mL sterile serological pipettes, or autopipettes with disposable sterile tips

Source cultures

The species cultured were originally collected by scientists from the Western Australian Government Department of Water and Environmental Regulation from wild stocks from East Brook (Warren River catchment). Source cultures of *Tetradesmus obliquus, Chlamydomonas oviformis, Chlorella* sp. and *Scenedesmus* sp. (Southwest Australian isolates) may be obtained from Ecotox Services Australia, 27/2 Chaplin Dr, Lane Cove NSW 2066, ph (02)9420-9480, info@ecotox.com.au.

Preparation of mass culture media

Axenic starter cultures are used to inoculate fresh autoclaved 100 mL liquid media cultures in 250 mL conical flasks, which when they have reached exponential growth phase, are used to inoculate more liquid cultures. Re-culturing into fresh media is performed on the same day each week. When used for toxicity testing, the timing of microalgal culture inoculation is critical to ensure that the cultures are in their exponential growth phase. Cultures should be inoculated 4 to 7 days prior to testing from an axenic source, whether this is from liquid starter cultures, in-house agar plate cultures, or from the liquid cultures that are re-cultured on a weekly basis.

Preparation of nutrient stock solutions

Five stock solutions for USEPA media are prepared (Table E.2.1). Analytical grade reagents must be used to prepare the stock solutions, by weighing out the required weight of the reagent and making up to the correct volume with deionised water. The stock solutions are then transferred into 250 or 500 mL Schott bottles and stored at 4 °C for up to one year.

Table E.2.1: Stock solutions required for the preparation of USEPA media for culturing freshwater microalgae for toxicity testing

Stock #	Reagent (analytical grade)	Weight required (g) for a final stock volume of 100 mL	Final concentration (g/L)
1	$MgCl_2.6H_2O$	1.216	1.216
	$CaCl_2.2H_2O$	0.44	4.40
	$NaNO_3$	2.55	25.50
2	$MgSO_4$ .7H2O	1.47	14.70
3	K_2HPO_4	0.1044	1.044
4	$NaHCO_3$	1.5	15.00
5	H_3BO_3	0.01856	0.1856
	$FeCl_3.6H_20$	0.01598	0.1598
	$MnCl_2.4H_2O$	0.0416	0.4160
	$Na_2.EDTA.2H_2O$	0.03	0.3000
	$ZnCl_2$	weigh 0.1640 g and make up to 100 mL with deionised water using a volumetric flask. Add 200 µL of this solution to the Stock #5 flask	0.0033
	$CoCl_2.6H_2O$	weigh 0.0714 g and make up to 100 mL with deionised water using a volumetric flask. Add 200 µL of this solution to the Stock #5 flask	0.0014
	$Na_2[MoO_4].2H_2O$	weigh 0.0366 g and make up to 10 mL with deionised water using a volumetric flask. Add 200 µL of this solution to the Stock #5 flask	0.0073
	$CuCl_2.2H_2O$	weigh 0.0600 g and make up to 1000 mL with deionised water using a volumetric flask. Add 1 mL of this solution to a 10 mL volumetric flask and make up to 10 mL. Add 200 µl of this second stock to the Stock #5 flask	0.012mg/L
	Na_2SeO_4	Weight 0.0598 g and make up to 50 mL with deionised water using a volumetric flask. Add 200 µL of this solution to the Stock #5 flask.	0.002392

Preparation of culture media

The volume of media to be prepared depends on the number of flasks to be inoculated. 100 mL of media is dispensed into 250 mL conical flasks prior to inoculation. Usually, two cultures are required each week. The volume of media to be prepared is therefore 200 mL.

- two 250 mL conical flasks with bungs, foil and indicator tape and 1 L Buchner flask and filtration apparatus (glass funnel, base and rubber stopper) are autoclaved.
- 150 mL of deionised water is transferred to a 200 mL volumetric flask and 0.20 mL of each of the five stock solutions is added in order of stock number (Table E.2.1) using auto pipettors, ensuring a fresh tip is used for each stock solution to prevent cross-contamination. Additional deionised water is added to bring the level up to the 200 mL mark and the flask is inverted ten times to homogenise the contents.

- volume of media is recorded.
- laminar flow cabinet, the clamp for the filter and the technician's hands must be cleaned and sterilised with alcohol wipes.
- all alcohol wiped items, forceps, and filter paper are placed into laminar flow.
- laminar flow cabinet is closed, and UV light is turned on for a minimum of 15 minutes for sterilisation.
- the forcep tips are flamed and used to pick up a 0.22 µm nitro-cellulose acetate filter and position it on the filtering apparatus.
- USEPA media is filtered.
- the bung is removed from the first flask and approximately 100 mL of filtered USEPA media is transferred.
- the bung is placed in the flask and the process is repeated for the second flask.
- Flasks of filtered sterilised media are stored in the refrigerator at 4 °C for up to one week.

Procedure for inoculation of culture media

The culture flasks selected as the source culture should be bright green in colour (indicating exponential growth phase) with no signs of clumping, yellowing or poor growth.

- flasks containing the 100 mL media are removed from the refrigerator at least one hour prior to inoculation and placed in the laminar flow cabinet
- hands, the laminar flow, the pipettor and the conical flasks to be inoculated are cleaned and alcohol wiped.
- all alcohol wiped items are placed into laminar flow.
- laminar flow is closed and the UV light is turned on for a minimum of 15 minutes for sterilisation.
- the culture flask is alcohol wiped and placed in the laminar flow. The bung is removed from the first conical flask and approximately 1 mL is transferred from the culture flask, using an autopipette with a sterile tip or a sterile serological pipette.
- the bung is placed in the flask and the process is repeated for each 250 mL flask culture.
- inoculation details are recorded ensuring each culture is assigned a unique identifier, with the record citing the immediate source of the inoculum.

Incubation of microalgal cultures

The inoculated flasks are placed on a light table and maintained at 25 ± 1 °C or in an environmental chamber at 25 ± 1 °C, under continuous lighting with intensity between 4440 and 8800 Lux. The 100 mL flask is examined and vigorously swirled at least twice daily, ensuring that all settled algae are re-suspended. Any cultures exhibiting signs of clumping may indicate bacterial contamination and are discarded with details recorded. After 7 days incubation, healthy cultures are bright green, indicating exponential growth phase has been attained. These cultures can be used to inoculate the next batch of cultures in the following week.

APPENDIX E.3: REFERENCE TOXICANT TESTING AND ESTABLISHMENT OF CONTROL CHARTS FOR FOUR CHLOROPHYTE TESTS: *TETRADESMUS OBLIQUUS, CHLAMYDOMONAS OVIFORMIS, SCENEDESMUS* SP. AND *CHLORELLA* SP. (SOUTHWEST AUSTRALIAN ISOLATES)

Reference toxicant (positive control) testing is included each time a toxicity test is undertaken to provide a mechanism for checking test precision. Any deviation of a test result from previously established values may highlight issues with test organism health, test conditions or performance of test personnel. For microalgae tests, the IC50 estimate (the concentration that results in 50% inhibition in cell yield) for a reference toxicant is compared to previous IC50 estimates (derived under near identical conditions) using a control chart. The control chart presents the running mean of IC50 estimates ± 2 standard deviations (SD) and ± 3 SD. If the IC50 of a reference toxicant test falls outside of ± 2 SDs but within ± 3 SDs, an investigation of the cause is triggered. Culture conditions, test conditions, verification of reference toxicant test concentrations should all be investigated. If the IC50 for the reference toxicant falls outside of ± 3 SDs of the running mean, the result and all concurrent test results should be rejected.

Control charts ideally consist of 20 individual IC50 estimates but may be commenced and used with a minimum of five individual IC50 estimates (USEPA 1993). Data used to establish a control chart (reference toxicant: copper sulphate) for *T. obliquus, C. oviformis, Scenedesmus* sp. and *Chlorella* sp. using the 72 hour growth inhibition test (cell yield) are presented in Tables E.3.1 to E.3.4 and Figures E.3.1 to E.3.4.

The test concentration based on a series of range-finding tests were 6.3, 12.5, 25, 50 and 100 µg Cu/L for *Chlorella* sp. and *Scenedesmus* sp. and 6.3, 12.5, and 50 µg Cu/L for *T. obliquus* and *C. oviformis*. The test data generally represent an un-interrupted dose-response relationship, covering the range from no effects to zero survival and as such are considered suitable for the purpose of a reference toxicant test.

Table E.3.1: The 72 h IC50 estimates for *T. obliquus* growth inhibition test (cell yield) for reference toxicant tests using copper sulphate

Test #	72 h IC50 ± SD (µg Cu/L)	95% Confidence Limits
1	23.7 ± 1.0	20.4-26.6
2	17.8 ± 0.5	16.4-19.5
3	34.5 ± 1.6	28.5-38.6
4	24.9 ± 2.0	21.9-31.9
5	24.0 ± 1.6	21.1-31.0

Table E.3.2: The 72 h IC50 estimates for *Chlamydomonas oviformis* growth inhibition test (cell yield) for reference toxicant tests using copper sulphate

Test #	72 h IC50 ± SD (µg Cu/L)	95% Confidence Limits
1	35.6 ± 1.6	31.2-40.7
2	33.5 ± 2.2	25.3-40.2
3	38.6 ± 0.8	36.0-40.6
4	27.7 ± 4.2	17.4-39.0
5	32.5 ± 0.9	29.7-35.5
6	37.5 ± 1.2	34.1-40.8

Table E.3.3: The 72 h IC50 estimates for *Scenedesmus* sp. growth inhibition test (cell yield) for reference toxicant tests using copper sulphate

Test #	72 h IC50 ± SD (µg Cu/L)	95% Confidence Limits
1	20.6 ± 0.9	18.2-23.2
2	19.3 ± 0.3	18.4-20.3
3	20.2 ± 0.7	17.9-22.1
4	21.0 ± 0.4	19.8-22.2
5	31.6 ± 1.1	28.6-35.2
6	48.4 ± 4.3	40.9-65.8

Table E.3.4: The 72 h IC50 estimates for *Chlorella* sp. growth inhibition test (cell yield) for reference toxicant tests using copper sulphate

Test #	72 h IC50 ± SD (µg Cu/L)	95% Confidence Limits
1	59.5 ± 3.0	46.1-65.9
2	65.3 ± 7.6	38.2-80.2
3	39.5 ± 1.6	34.1-44.0
4	45.1 ± 7.4	31.2-71.5
5	43.7 ± 2.2	38.1-51.0

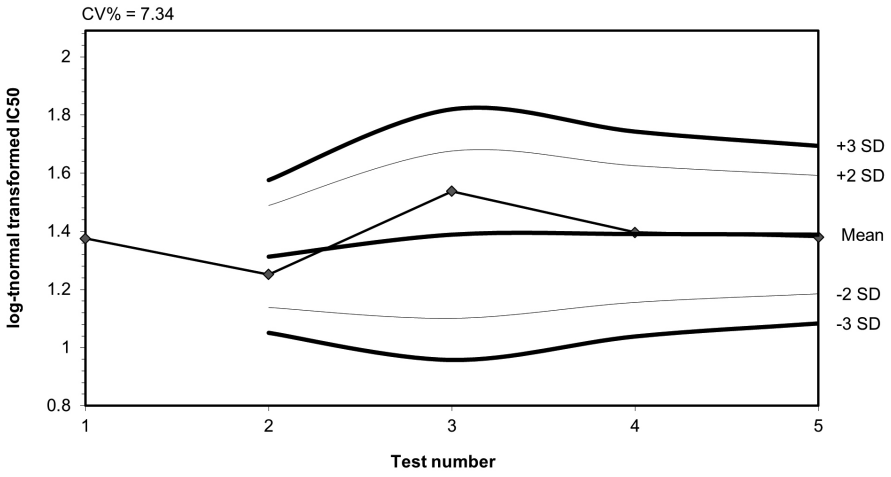


Figure E.3.1: Control chart using log-normal transformed 72 h IC50 estimates (µg Cu/L) for *Tetradesmus obliquus* growth inhibition test (cell yield) for reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

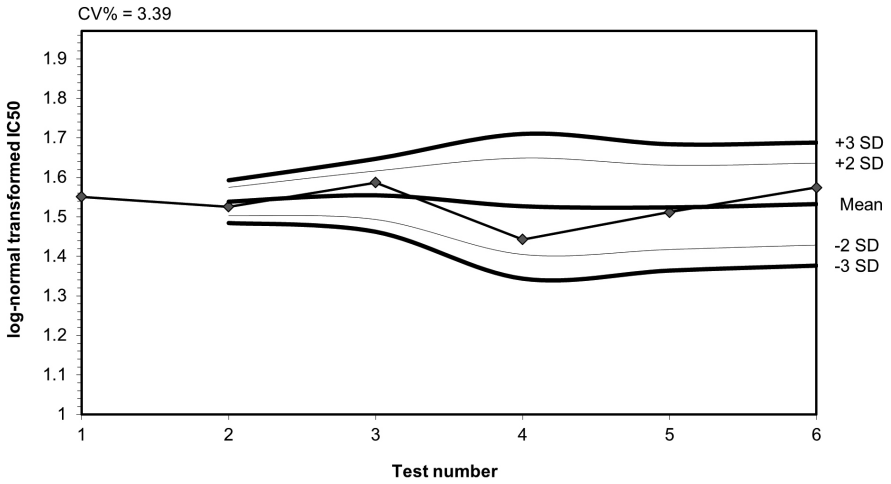


Figure E.3.2: Control chart using log-normal transformed 72 h IC50 estimates (µg Cu/L) for *Chlamydomonas oviformis* growth inhibition test (cell yield) for reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

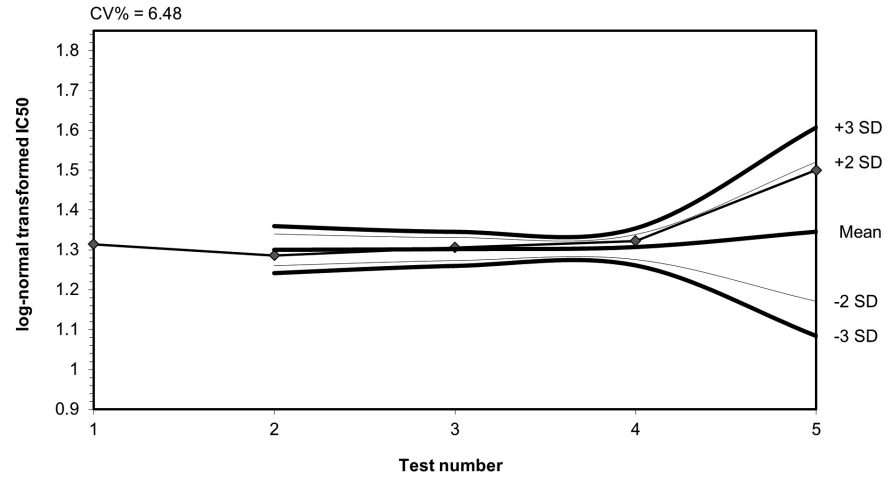


Figure E.3.3: Control chart using log-normal transformed 72 h IC50 estimates (µg Cu/L) for *Scenedesmus* sp. growth inhibition test (cell yield) for reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

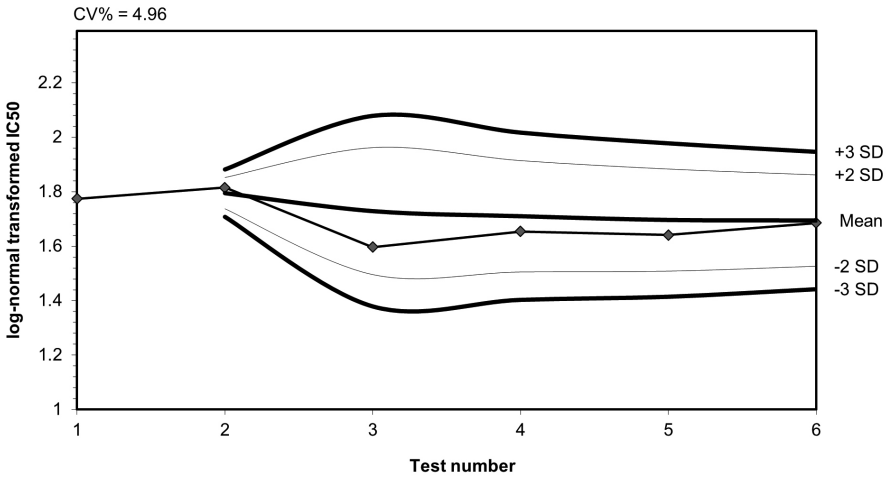


Figure E.3.4: Control chart using log-normal transformed 72 h IC50 estimates (µg Cu/L) for *Chlorella* sp. growth inhibition test (cell yield) for reference toxicant tests using copper sulphate (determined using Toxcalc – Tidepool Scientific Software, v5.0.23)

APPENDIX F: PREPARATION OF GP-2 SALTS FOR TEST DILUENT AND CONTROL WATER

Table F.1: The weight of analytical grade reagents combined to prepare GP-2 Artificial Salts, used for the salinity adjustment of samples for toxicity testing (USEPA 2002b)

Compound	Amount Required (g)
NaCl	420.6
Na_2SO_4	70.4
KCl	12.2
KBr	1.76
$Na_2B_4O_7.10H_2O$	0.68
$MgCl_2.6H_2O$	190.0
$CaCl_2.2H_2O$	26.4
$SrCl_2.6H_2O$	0.4
$NaHCO_3$	3.4