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 6

2020

AUSTRALASIAN BULLETIN OF ECOTOXICOLOGY & ENVIRONMENTAL CHEMISTRY

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STANDARDISED CHRONIC TOXICITY TEST PROTOCOLS AND CULTURING METHODS FOR A SUITE OF TROPICAL FRESHWATER SPECIES

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ABSTRACT

This paper presents a compilation of ecotoxicological test protocols developed at the Environmental Research Institute of the Supervising Scientist (*eriss*) in Australia. It provides detailed methods for seven tropical species: the green alga *Chlorella* sp., duckweed *Lemna aequinoctialis*, cladoceran *Moinodaphnia macleayi*, coelenterate *Hydra viridissima*, gastropod mollusc *Amerianna cumingi*, freshwater mussel *Velesunio* spp., and the teleost fish *Mogurnda mogurnda*.

Keywords: aquatic ecotoxicology; Cladocera; alga; duckweed; *Hydra*; mussel; gastropod; fish.

TABLE OF CONTENTS

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INTRODUCTION

This compilation of ecotoxicological test protocols has been developed at the Environmental Research Institute of the Supervising Scientist (*eriss*) over the past 40 years. The objective of these tests is to determine the concentration of a test chemical or sample that affects a specified endpoint relating to the health of a particular aquatic organism. This information can then be used alongside toxicity data from other aquatic organisms (from different taxonomic groups and trophic levels) to estimate the risks to the freshwater environment associated with releasing that particular chemical or sample.

Recognising the inadequacies of using default water quality guidelines to protect specific aquatic ecosystems (ANZG 2018), these toxicity tests were developed to i) create site-specific water quality guidelines for the highly valued, world heritage-listed Kakadu National Park of the Alligator Rivers Region, Northern Territory, and ii) specify a dilution of any given waste water required to provide a 99% protection level for aquatic organisms of tropical freshwater systems in this region. Much of the research carried out for the species contained in this issue has been a regulatory requirement for the protection of the Alligator Rivers Region from the effects of mining activities.

Given the extensive period that has passed since the original publication of many of these methods (Riethmuller et al. 2003), the methods for both culturing and testing have undergone major changes and improvements. There are also newly developed tests for mussel and fish species. As such, the objective of this compilation of methods was to communicate the complete set of our most up-to-date methods and have them easily accessible in one location, in support of open science (Moermond et al. 2016, SETAC 2019). It is hoped that these methods may be used and adapted more broadly by other research groups working with aquatic organisms.

Historical and recent references for all the test protocols are outlined (Table 1), along with the culturing requirements for each test species. Recipes for media and food, and more detailed analytical procedures associated with the tests or culturing can be found in the Appendices. The authors can be contacted to provide datasheets for each of the test protocols.

Table 1. Current suite of tropical test species local to the Alligator Rivers Region and the studies that have contributed to the development of these test methods.

Test organism	Duration	Test endpoint	Key references
Chlorella sp. (green alga)	72 h	Cell division (doublings/d)	Stauber et al. (1989) Franklin et al. (1998) Franklin et al. (2002) Franklin et al. (2005) Wilde et al. (2006) Pease et al. (2016a)
Lemna aequinoctialis (duckweed)	96 h	Growth (surface area)	Hogan et al. (2010) Pease et al. (2016b)
Moinodaphnia macleayi (cladoceran)	5-6 d	Reproduction (3 brood)	McBride et al. (1991) Hyne (1991) Hyne et al. (1993) Rippon & le Gras (1993) Hyne et al. (1996)
Hydra viridissima (green hydra)	96 h	Population growth	Riethmuller et al. (2000) Riethmuller et al. (2001) Riethmuller et al. (2003)
Amerianna cumingi (gastropod)	96 h or 14 d	Reproduction (egg number)	Houston et al. (2007) Hogan et al. (2010)
Velesunio angasi (mussel)	14 d	Growth (shell length)	Kleinhenz et al. (2019)
Mogurnda mogurnda (northern trout gudgeon)	7 d	Growth (length)	Riethmuller et al. (2000) Cheng et al. (2010) Pease et al. (in press)

GENERIC METHODOLOGY

CULTURE WATER AND TEST DILUENT

There are two types of water routinely used for toxicity testing at *eriss*: natural creek water and a synthetic version of that water. The aim of the test and the test organism determines which type of water is used. Some tests may need to be conducted with minimal organic matter present; synthetic water is ideal for this purpose. With regards to the test organism, the cladoceran *M. macleayi* and gastropod *A. cumingi* cannot be tested in synthetic water because it is known to result in poor health and survival, and thus must be tested in natural creek water. For culturing, depending on the organism (refer to the sections for each species), either natural creek water, filtered Darwin tap water or bore water (Jabiru Field Station) is used.

Natural creek water

Natural water is collected at least monthly from two reference sites upstream of waste water discharge from Ranger mine; from Magela Creek (latitude 12° 40' 28" S, longitude 132° 55' 52" E) in the wet season and from Bowerbird Billabong (latitude 12° 46' 15" S, longitude 133° 02' 20" E) via helicopter access in the dry season. Water is collected in acid-washed 10-L or 20-L plastic containers as close as possible to the start of a test. After transport to the laboratory (approximately 3 h travel), the water is filtered (2.5 µm) (SupaPore PP Capsules, Amazon Filters) within 48 h of collection. The water is stored at 4°C for a maximum period of four weeks before being discarded. Magela Creek water (MCW) cannot be stored longer because of its poor buffering capacity and deterioration of water quality in storage.

Synthetic soft water

Synthetic soft water (SSW) has been designed to simulate the inorganic composition of water from Magela Creek and other sandy, braided streams characteristic of the Northern Territory (Appendix Table A1). As such, this water is very soft and slightly acidic with low buffering capacity. As SSW does not contain organic material, it enables test organisms to be exposed to contaminants under conditions in which both the sensitivity of the organism and the bioavailability of metal contaminants are maximised (Markich et al. 2000, Trenfield et al. 2011; 2012). This enables conservative toxicity estimates to be generated. Synthetic soft water is prepared by adding a range of analytical grade chemical salts (Appendix Table A2) to high purity Milli-Q® water and this is left to equilibrate overnight. Following equilibration, pH is usually between 6.5 and 6.7 and is adjusted to pH 6.0 ± 0.15 using 0.05M H_2SO_4.

Filtered Darwin tap water

Filtered Darwin tap water (FDTW) is a soft water with low conductivity (pH ~7, EC =75 µS/cm, hardness ~26 mg/L as $CaCO_3$), which is filtered to remove solids (cartridge filtration; 20, 5 and 1 µm) and also filtered through activated carbon to reduce concentrations of trace metals. After filtration, the water is stored in temperature-controlled holding tanks (~27°C) and it passes through PVC piping into the aquaculture laboratory aquaria as needed. This water is used for culturing the snail and fish species as the volume of water required to maintain these species on flow-through systems is too large to use MCW. The snails are also cultured in tubs of bore water at the Jabiru field station. The bore water has a pH of 7 to 8 and is diluted with reverse-osmosis water to maintain an electrical conductivity (EC) of around 100 µS/cm. While the fish, alga, cladoceran and *Hydra* are the only species that have been tested in hard water (up to 375 mg/L $CaCO_3$), with no adverse

effects observed due to hardness (Riethmuller et al. 2000, Markich et al. 2005), it is likely the other species in the suite could be cultured and tested without detriment in moderately-hard tap water if necessary.

STOCK SOLUTIONS OR EFFLUENT

Stock solutions are prepared using analytical-grade reagents in high-purity water and stored in acid-washed polyethylene containers which are refrigerated at 4°C. If whole effluent is being assessed, it is collected as close as possible to the test start and stored under the same conditions as stock solutions. The method of collection used depends on the sampling site and conditions. For example, for larger volumes where a submersible pump can be used, it is preferable to pump water into collection containers. For smaller volumes (e.g. up to 100 L), provided the water level at the collection point is high enough and that it is safe to do so, water can be collected simply through the submersion and filling of containers. With regards to the collection of highly contaminated water, the collection container can be lowered into the water using a bucket/rope pulley system by an operator wearing gloves, so that there is no direct contact between the operator and the effluent.

TEST SOLUTIONS

Test solutions are prepared by diluting a stock solution or effluent with diluent water. Test solutions are generally prepared on the day of the test start unless a period of equilibration is required. In that case, test solutions may be prepared the day prior. For contaminants that are not volatile or prone to degradation, we have found it suitable to prepare sufficient test solution in bulk at the start to last the entire test duration (rather than preparing new test solutions on a daily basis). Any loss of contaminants (for example, metal adsorption to test containers) is accounted for by calculating an arithmetic mean of measured concentrations of the contaminants at the start and end of the test. This approach minimises chemistry costs as test solutions do not have to be analysed on a daily basis as they ideally would if test solutions were prepared each day. When testing with contaminants that are volatile (e.g. ammonia) or degrade (e.g. hydrocarbons or light-sensitive compounds), it is recommended that test solutions are prepared each day, or if necessary, initial trials are run to determine the maximum acceptable period between preparation of test solutions (as this may be less than 24 h for some contaminants).

REFERENCE TOXICITY TESTING

Reference toxicity tests for uranium (a contaminant of concern for the Alligator Rivers Region, Northern Territory, Australia) are run quarterly. Due to the abundance of toxicity data that *eriss* has generated for uranium exposures to most of these test species (~40 y of testing), it provides a reliable benchmark (EC10s and EC50s) against which the consistency of the organism's sensitivity can be assessed. Figure 1 shows how the data can be used to recognise a change in sensitivity of a test organism. Lower and upper warning limits, calculated as two standard deviations (SD) of the mean, can be used to provide an alert that the test organism is not responding to a contaminant as expected. Such an event may lead to investigations into all aspects of the test system, including culture health, contamination, potential errors in the way the test was conducted, or the possibility of culture fatigue, where an organism has been cultured for a long period in a laboratory environment. The lower and upper confidence limits are calculated as three SD of the mean and if a response falls outside of these limits, it is deemed unacceptably inconsistent with historical data. Such a response would result in any associated data generated at the same time for other contaminants being considered unsuitable for publication. If such a response continued to occur, attempts would be made to replenish the laboratory culture with organisms newly collected from the field.

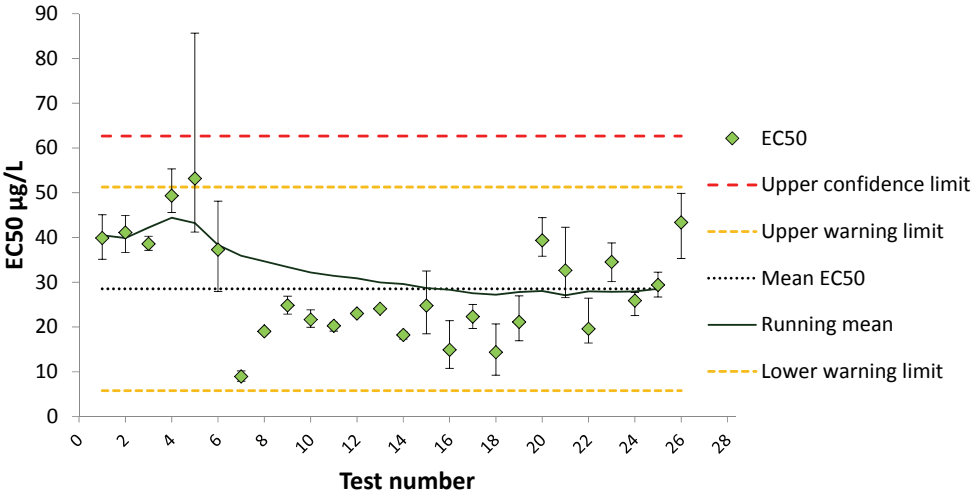

Figure 1. Reference toxicity test plot showing 50% effect concentrations (EC50s) for *Chlorella* sp. exposed to uranium in synthetic soft water using the method developed at *eriss* in 2016. Data are shown for the period 2016-2019.

Where necessary, assays of other reference toxicants (such as copper) may be run alongside a toxicity test. In addition to the reference toxicity testing program (unpublished data), toxicity data have been published for U in local Magela Creek water of low hardness (2 to 4 mg/L $CaCO_3$, Table 2). Data have also been generated for Cu exposure to some of these species (Table 2).

Table 2. EC50s (95% confidence limits) for local, tropical test species exposed to copper (Cu) and uranium (U) in Magela Creek water (unless specified otherwise).

Species	EC50 µg/L (95% CL)			
	Cu	Reference	U	Reference
Chlorella sp.	1.5	Franklin et al. (2002)[a]	70 (65-74)	*eriss* unpublished[b]
Lemna aequinoctialis	16.2 (15.5-16.9)	Charles et al. (2006)[a]	1435 (NC)	Hogan et al. (2010)
Moinodaphnia macleayi	23 (17-24)	Orchard et al. (2002)	39 (35-44)	*eriss* unpublished[b]
Hydra viridissima	5.5	Riethmuller et al. (2000)[a] Unpublished data (2020)	79 (73-85)	Trenfield et al. (2011)
Amerianna cumingi	ND		278 (NC)	Hogan et al. (2010)
Velesunio angasi	6.9 (NR)	Kleinhenz (2019)	304 (NR)	Kleinhenz (2019)
Mogurnda mogurnda	ND		1124 (1016-1246)	Pease et al. (in press)

[a] exposure in synthetic soft water, [b] contact primary author for access to unpublished *eriss* data, NC = not calculable, NR = confidence limits not reported, ND = no data.

TESTING AREA

The preparation of test solutions should be carried out in an area with ample room and that is free of contamination from harmful vapours, dust or disturbance. Before commencing any part of the test procedure, workers should wash hands and arms with fragrance-free soap and rinse with tap water to avoid introducing contamination during daily observations and water exchanges. Disposable gloves should also be worn.

PREPARATION OF EQUIPMENT

All equipment used to hold test organisms or media is made of chemically inert materials. All plastic and glassware is soaked in 5% HNO_3 for 24 h before undergoing a detergent wash (Neodisher Laboclean FLA, GKE Australia) and rinsed in a laboratory dishwasher using reverse osmosis (RO) water. All glassware except volumetric flasks is silanised with 2% dimethyldichlorosilane in 1,1,1-trichloroethane (Coatasil, AJAX) to reduce metal adsorption to the glass.

STANDARD EQUIPMENT

The following equipment is used across all toxicity tests. Any specialised equipment will be mentioned in the species-specific method sections.

- light-tight constant temperature incubator
- ultra-pure water purification system (producing water of ~18 MΩ/cm resistivity, e.g. Milli-Q® water)
- refrigerator (set at 4 °C) for storage of test solution and stocks
- pH, electrical conductivity and dissolved oxygen meters for measuring water parameters
- 120 mL tall-form HDPE vials for water parameter samples
- automatic adjustable pipettes/dispensers (100 µL, 1 mL, 5 mL, 10 mL and 50 mL) and disposable pipette tips
- temperature logging system (we use a Testo Saveris 2 monitoring system)
- chemicals and reagents
- A-grade volumetric flasks
- magnetic stirrer and stir bars
- analytical balance and weigh boats
- random number tables
- borosilicate test vessels (2 L beakers, 250 mL Erlenmeyer flasks with aluminium foil caps)
- plastic test vials (40 mL polyethylene (Cospak #12) vials with screw caps, 90 mm diameter disposable Petri dishes with lids, 175 mL round containers with lids)
- 500 mL to 5 L polyethylene containers (to hold treatment solutions)
- plastic/glass 2 mL pipettes
- binocular dissecting microscope
- clear Perspex trays to hold test vessels (with position numbers 1 to 24 marked)
- laboratory warming trays
- sterile 30 or 50 mL syringes and 0.45 µm syringe filters for sampling chemistry

- sterile polyethylene vials (15, 50, 100 and 250 mL) with plastic screw caps for analytical chemistry samples
- polyethylene beakers for test solution making and rinsing during snail tests
- microscope-mounted camera for photographing test individuals
- ultra-pure 69% HNO_3 for chemistry sample preservation and analytical reagent grade 70% HNO_3 for acid baths
- NaOH and H_2SO_4 for test water pH adjustment
- phosphate-free dishwasher detergent: Neodisher Laboclean FLA and Neodisher Z (Dr Weigert)
- Decon Neutracon (Neutrad) detergent
- 10 g/L MS222 stock solution for euthanasing fry

PHYSICO-CHEMICAL ANALYSES OF TEST WATERS

Physico-chemical analyses are undertaken on various culture and test waters in order to:

- ensure that only high-quality water is used for culturing and testing
- check the accuracy of test solution chemical concentrations
- understand the effects of natural water parameters (e.g. organic carbon) on toxicity
- ascertain whether contamination of test solutions has occurred within the laboratory.

Physical parameters of water samples (pH, dissolved oxygen (DO) and electrical conductivity (EC)) are measured either at the time of collection or immediately following transport of the natural water to the laboratory. For test waters, physical parameters are measured either at the commencement and termination of tests (*Chlorella* sp. and *L. aequinoctialis*) or measured daily on new and 24-h-old water (all other species). It is important to note that physical water parameters at the start of the test should be measured prior to the test starting. This allows instances of contamination to be identified and test solutions can be prepared again if necessary.

For a test to be considered valid, the following physical parameter acceptability criteria must be met for the control treatment:

- the recorded pH does not exceed 1 pH unit of change between new and old waters
- the DO concentration must be >70% in old waters throughout the test period
- the EC for each test solution is within 10% of the values obtained at test start.

Where these criteria are met by the control but are not by a particular treatment, the test is considered valid. However, it is recommended that the affected treatment is excluded from the final analyses.

For chemical analyses of diluent, stock solutions or test solutions, the standard regime is:

- Monthly *total* metal analyses (Al, As, B, Ba, Br, Ca, Cd, Co, Cr, Cu, Fe, Hg, I, K, Li, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sr, U and Zn) on filtered and unfiltered Darwin tap water and fish tank water. Samples for total metals are acidified to 1% HNO_3 using ultra-pure concentrated HNO_3 and submitted for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analyses, and if results are higher than expected (Appendix Table A3), water samples are collected again and submitted for re-analysis. If re-analysis still shows the presence of elevated metals, it is an indication that the

activated carbon in the carbon filters is exhausted, allowing metals to pass through filtration. Replacement of the activated carbon should resolve this problem.

- Monthly alkalinity, Dissolved Organic Carbon (DOC) and total metal analyses on both 2.5-µm filtered and unfiltered natural Magela Creek water (at time of collection).
- Filtered (<0.45-μm fraction) and total metals for all test waters in spiked toxicant tests or effluent exposures. For each test, a 'blank' sample is collected (Milli-Q® water directly from the purification system) along with a 'procedural blank' sample. The 'procedural blank' sample is taken at the time that test solutions are prepared, by passing Milli-Q® water through all of the steps involved in test solution preparation. In doing so, the Milli-Q® water comes in contact with the inner surface of all components involved in the test system such as volumetric flasks, test solution bottles, dispensers and test containers such as Petri dishes). This provides an indication of any contamination introduced through the preparation of test solutions. Following acidification to 1% HNO_3 using ultra-pure concentrated HNO_3, blanks, procedural blanks and control samples are analysed for the suite Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, SO^{2-}, Se, U and Zn. All other test solutions are analysed only for the key contaminant (if known).
- NO^{3-} and PO^{3-} are measured for the blank, procedural blank and control water from *Chlorella* sp. and *L. aequinoctialis* tests to determine the accuracy of nutrient additions.
- Total ammonia nitrogen (TAN) is measured for tests where TAN is a contaminant of interest, and for all fish and snail tests. For fish and snail tests where the generation of waste by the test organism could compromise water quality of the test solution, we have found it appropriate to check TAN on a daily basis and ensure it is ≤0.5 mg/L for the fish and ≤1.0 mg/L for the snails across the test period (at ~pH 6 and 28°C).

The above sampling regimes are only a guide and the water chemistry regime for any particular project will depend on project funding and the questions that need to be answered.

RANDOMISATION AND AVOIDING BIAS

Random number tables are used to ensure the random placement of replicates during tests. This is an important part of the experimental design. We use the Research Randomizer website (https://www.randomizer.org/) to generate random number tables. Replicates are re-randomised daily using a new set of random numbers.

ACCEPTABILITY OF TEST DATA

A test is considered acceptable if the following general criteria are met (refer to respective sections on individual species for species-specific requirements):

- The recorded temperature of the incubator remains within the prescribed limits (the desired temperature for a particular species ± 1°C).
- The growth rate or reproduction of the control treatment is within an acceptable range for the species and >80% of the control individuals survive.
- There is <20% co-efficient of variability (CV) in the control growth rate or reproduction.
- The physical parameters of the test waters meet previously described acceptability criteria.
- The result of associated reference toxicity testing is within the warning limits for that particular species (as described in Reference Toxicity Testing).

ANALYSES OF TEST DATA

Effect concentrations (ECxs; generally EC10s and EC50s) can be generated using statistical software that is designed to analyse toxicity data, such as CETIS or ToxCalc. For each treatment, the response of 2 or 3 replicates is averaged and the mean is expressed as a function of the control response (as a percentage of control response). Toxicant concentration is log transformed in all analyses. Data are screened for normality and homogeneity using the Shapiro-Wilk Normality Test and Bartlett's test for Equality of Variances. Where data are non-normal, a weighting can be applied to the data, such as Box-Cox or gamma, depending on whether the data are discrete or continuous (Mooney et al. 2018). If data are still non-normal after applying an appropriate weighting, it is likely there is a greater underlying issue with the data. ECxs are derived for each organism and test water using non-linear logistic regression. Sigmaplot 14 is also used to generate response curves (usually using sigmoidal or logistic models, depending on which provides the closest fit to the data) from which any ECx (and the associated CLs) can be derived.

TEST ORGANISMS

CHLORELLA SP.

The unicellular freshwater green alga *Chlorella* sp. was originally isolated from Magela Creek within Kakadu National Park in 1991 and has since been maintained at the *eriss* facility.

ORGANISM CULTURE

Algae are cultured at 75 µmol photons PAR/m^2/s on a 12:12 h light:dark cycle using cool white fluorescent lighting. A light meter is used to determine light intensity of both the culture and test incubators. Cultures are maintained in 100 mL volumes in 250 mL borosilicate Erlenmeyer flasks in constant temperature growth cabinets kept at 29 ± 1°C. The cultures are placed on an orbital shaker at a speed of 150 rpm to keep cells in suspension and maximise their exposure to light, nutrients and gases. Each week, ~2 mL of *Chlorella* sp. is aseptically transferred to new growth medium. Fresh growth medium (MBL) is prepared each month (Appendix B).

PRINCIPLE OF THE TEST

Cultured *Chlorella* sp. within the exponential growth phase (approximately 4 to 5-d-old) is exposed to either a contaminant across a concentration range or a series of dilutions of an effluent. The exposures are conducted over 72 h with cell counts taken at 0 h, 48 h and 72 h to calculate a cell division rate. The concentration-response relationship is modelled using non-linear regression and toxicity estimates are derived from this model. The endpoints measured are the 72-h EC10 and EC50 which are the effective contaminant concentrations or effluent dilution at which there is a 10% or 50% reduction in algal growth rate compared to the controls (unexposed replicates). This test is based on international standards OECD (2011) and USEPA (2002).

TEST SOLUTIONS

All test waters, regardless of diluent, need to be supplemented with nutrients to promote algal growth in controls that is sufficient to measure a toxicological response in test exposures. A buffer (1 mM HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is used to minimise pH drift throughout the test period. Trials by Trenfield (2012) demonstrated unacceptable pH shift (from pH 6.1 to pH 7.7) when a buffer was not used and the pH of test solutions was manually adjusted every 24 h. Those trials demonstrated that test solutions would have to be manually adjusted every 2 to 4 h of a 72 h exposure in order for pH to remain within an acceptable range (± 0.2 pH units), and this method was therefore considered to be impracticable. Test solutions are prepared by the following:

1. A total of 5 L of nutrient-supplemented test water is made (using either Magela Creek water or SSW) to contain final nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations of 3.63 mg/L and 34.5 µg/L, respectively (using $NaNO_3$ and KH_2PO_4), and a final HEPES concentration of 1 mM. These nutrient concentrations, which were trialled and determined by Pease et al. (2016a), are 4-fold less than those used in the original method reported by Riethmuller et al. (2003). These lower nutrient concentrations support sufficient growth of the algae at a lower starting cell density (10^3 cells/mL) compared to the higher nutrient concentrations required for the cell density used in the original protocol (10^4 cells/mL).
2. For each treatment, 500 mL of the above test water is allocated.
3. For each treatment, 50 mL aliquots are dispensed into three Erlenmeyer flask replicates. The remaining test solution for each treatment is used for the validation of toxicant and nutrient concentrations and water quality measurements. The flasks of test solution are left to warm in the test incubator for at least 1 h before starting the test.

TEST PREPARATION

1. Approximately 1 hour prior to the desired test start time, *Chlorella* sp. cells are harvested from a 4 to 5 d-old stock culture in the exponential growth phase. The algal cells are centrifuged in a 50 mL plastic centrifuge tube at 1310 *g* for 7 minutes. The supernatant is then decanted and the cell-pellet resuspended in approximately 30 mL of ultra-pure water by vortexing. The washing process is repeated three times to ensure that the nutrient-enriched culture medium is removed.

2. After three rinses, the pellet is resuspended in approximately 40 mL of Milli-Q® water. A 1:100 dilution of this cell suspension is prepared (250 µL into 25 mL of Milli-Q® water). A cell count is carried out on a 25 µL sub-sample of the 1:100 dilution. Cell counts can be done manually on a compound microscope (Appendix C) or through more automated techniques using a Coulter counter or a flow cytometer. At *eriss*, a BD Accuri C6 flow cytometer is used.

3. The cell count is multiplied by the dilution factors (40 times for the 25 µL subsample taken and 100 times for the initial 1:100 dilution) to give the density of the washed cell suspension in cells/mL. The desired cell density in each 50 mL aliquot of test solution (3 x 10^3 cells/mL) is divided by the cell density of the inoculum to calculate the volume of the inoculum to add to each test flask (see example below). Two additional 'cell density' flasks are also inoculated and counted only at test start to provide a starting test cell density (i.e. 3 x 10^3 cells/mL).

Example:

Algal counts: = 738, 728, 714, 657
Mean = 709.25
Algal count adjusted by dilution factor (x100): = 70925 cells in 25 µL
Cell density of inoculum (cells/mL): 70925 x 40 = 2.84 x 106
Desired cell count for a 50 mL flask: 50 mL x (3 x 103)
Desired cell count/cell density of inoculum = 150 x 103/2.84 x 106 = 0.0528 mL
= 53 µL added to each test flask of 50 mL test solution
Tests are performed with three replicates per treatment. We have found that, given the low variability in cell division rate between control replicates (~2.3% CV), this design may be changed to two replicates per treatment if required, provided this does not result in >10% CV within each treatment. This then allows for more toxicant concentrations to be tested.

One flask containing test solution but no algal inoculum is used to determine the background levels of fluorescence in non-algal particles. If the diluent used across all treatments is similar in composition, one blank flask can be used for all treatments.

TEST PROCEDURE

Day 0

1. Labelled test flasks are removed from the incubator and transferred to a bench for inoculation. Test flasks are inoculated with the exact volume of algal inoculum, beginning with the control replicates and working up the concentration gradient. The flask of inoculum should be swirled each time before algae are pipetted.

2. The test flasks are then placed randomly on the shelves in a light-tight constant temperature growth chamber at 29 ± 1°C on a 12:12 h light:dark cycle at 100 to 150 µmol photons PAR/m^2/s.

Days 1-3

3. Each flask is gently swirled by hand, twice daily throughout the test to avoid gas limitation, approximately six times in both clockwise and anti-clockwise directions.

4. The cell density in each flask is determined at both 48 h and 72 h.
5. The internal base of each flask should be gently scraped using a cell scraper to resuspend any settled algal cells. A 1 mL sub-sample is then taken and placed in a 5 mL Potter-Elvehjem tissue grinder and homogenised using the Teflon pestle to break down any clumps of algal cells. 500 µL of this sample is then placed in a clean 2 mL sample tube and placed on the flow cytometer sampling stage.
6. Three 25 µL sub-samples are counted and an average taken of the number of events that occur within the gated region that represents algae auto-fluorescing in the red range. This process is then repeated for each replicate, with the blank flask containing no algae being measured first to determine background levels of fluorescence.
7. The flow cytometer is back-flushed between treatments. The homogeniser, pestle and sample tubes are also rinsed with ultra-pure water between treatments.

ANALYSIS OF TEST DATA

The growth rate of the algae in each flask is calculated using linear regression analyses (see Excel worksheet in Appendix D). A regression is plotted for ln cell density vs. time (h) to determine the slope of the line for each flask, which is equivalent to the cell division rate per h (µ) for each treatment. Doublings/d are calculated by multiplying the cell division rate by 24 (number of hours in a day) then by the constant of 1.443. The algal population growth constant is based on calculations provided by Dr Merrin Adams at CSIRO Land and Water and are shown in Appendix E. The growth rate of the control algae must be within the range 2.0 ± 0.3 (mean ± SD) doublings/d to be considered acceptable and for analyses to be performed. Growth rate data are then analysed as described in the Generic Methodology section.

LEMNA AEQUINOCTIALIS

Lemna aequinoctialis Welwitsch (Lemnaceae, Spathiflorae) is a small aquatic, flowering macrophyte commonly known as duckweed. The duckweeds are primary producers and a source of food for water fowl, fish and small invertebrates and they provide habitat for many small organisms. Their small size and fast growth rates make them ideal for testing in the laboratory. They were originally collected from surface waters of Kakadu National Park in the Northern Territory, Australia in 1997 (Yellow Water Billabong, Cooinda; latitude 12° 52' S, longitude 132° 30' E).

ORGANISM CULTURE

An axenic culture is maintained at *eriss* in 50% modified Hoagland's E and K medium (CAAC) at 29 ± 1°C on a 12:12 h light to dark cycle (115-125 µmol photons PAR/m^2/s). The CAAC medium, developed by the CSIRO Centre for Advanced Analytical Chemistry (currently known as the Centre for Environmental Contaminants Research) is a variation of Hoagland's E (Cleland and Briggs 1969) and K medium (Maeng and Khudari 1973). The recipe is shown in Appendix F.

One litre of CAAC medium is prepared at least monthly. For routine culture maintenance, 2 x 100 mL flasks of medium are inoculated with ~10 to 15 plants (each with 3-4 fronds) each week. Inoculations are carried out in the laminar flow cabinet.

Older cultures are retained for several weeks as backup in case contamination occurs. The parent culture should have 70 to 100% cover when used to inoculate the new culture (this typically occurs 7 d after inoculation, Figure 2).

Lemna selected for testing must be free of overt disease and gross morphological deformity, where each plant has healthy green fronds and root system. A suitable test plant is one that has three fronds only; two mature fronds and one slightly smaller, less-developed frond (Figure 2).

PRINCIPLE OF THE TEST

A standard number of vegetatively reproducing *Lemna* plants are exposed to a range of concentrations of a toxicant for 96 h under controlled conditions. An increase in biomass is calculated based on the number of fronds counted at the end of the test, and the surface area of the plants. Photographs are taken at the start and end of the test and increase in surface area is calculated using image analysis software (e.g. ImageJ, Threshold colour add-on). The endpoints measured are the 96 h EC10s and

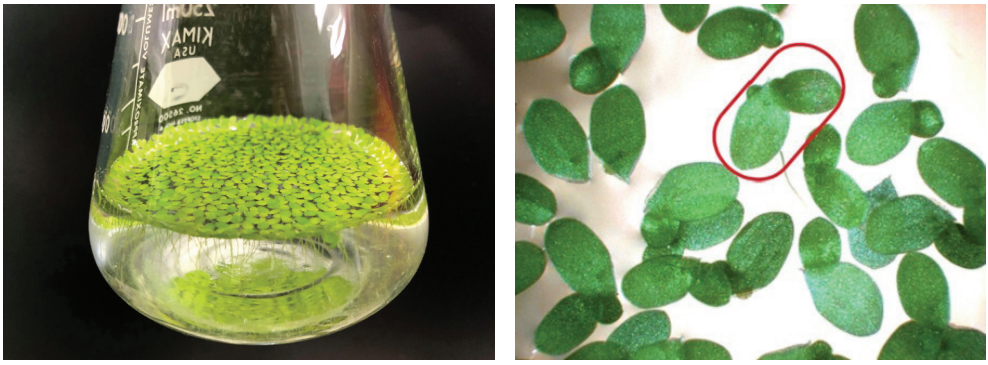

Figure 2. Left: A one-week-old *L. aequinoctialis* culture with 100% cover. Right: Correct stage of *L. aequinoctialis* for test start.

EC50s, which are the effective contaminant concentrations or effluent dilutions at which there is a 10% or 50% reduction in the growth rate of *Lemna* (based on surface area and frond number), compared to the controls (unexposed replicates).

This protocol has been modified from the protocol described in Riethmuller et al. (2003) to include a more sensitive secondary endpoint of surface area (SA), along with frond number, and a reduction in the concentration of nutrients added to the test diluent (reduced from 3.0 mg/L NO_3^- and 0.3 mg/L PO_4^{3-} to 1.0 mg/L NO_3^- and 0.1 mg/L PO_4^{3-}). As described by Pease et al. (2016b), nutrient concentrations were reduced in order to maximise the sensitivity of the *Lemna* to contaminants, resulting in a mean EC50 of 260 µg/L U compared to 8960 µg/L in the original test diluent (based on a SA endpoint). The increased sensitivity is likely due to the reduced concentration of potential metal ligands in the test system. The original protocol of Riethmuller et al. (2003) was based on international standards (OECD 2006; ASTM 1992).

TEST SOLUTIONS

The diluent water used in testing is enriched with nutrients in order to promote healthy *Lemna* growth. Individual NO_3^- and PO_4^{3-} stocks are made up in 1 L of Milli-Q® using 36.08 g/L KNO_3 and 6.5999 g/L KH_2PO_4. Nutrient-enriched water should be made in batches to ensure nutrient homogeneity between treatments. Five litres of diluent is made up by adding 226 µL KNO_3 and 109 µL of KH_2PO_4 to make a solution of 1.0 mg/L NO_3^- and 0.1 mg/L PO_4^{3-}. One litre of test solution is made per treatment using the nutrient-enriched diluent, and for each treatment, 100 mL is dispensed into each 250 mL Erlenmeyer flask replicate. The remaining test solution is subsampled for chemistry analyses and (metal and nutrient concentrations), and water quality measurements.

TEST PREPARATION

Additional flasks of *Lemna* may need to be inoculated the week leading up to a test so that there are sufficient plants that are of the correct 3-fronded stage to start a test. It is recommended that 2 to 4 extra flasks are inoculated, depending on the size of the test.

TEST PROCEDURE

Day 0

1. Prepare test solutions, check pH, adjust if necessary (we use a pH of 6 ± 0.1 for *L. aequinoctialis*) and leave for at least 1 h to equilibrate.
2. Perform water quality checks and collect samples for chemical and nutrient analysis.
3. Dispense 100 mL aliquots of test solution into three labelled Erlenmeyer flasks per treatment and 50 mL into a water parameter vial; place in incubator to warm to test temperature.
4. *Lemna* plants at the correct stage are removed from the culture media and placed in a crystallising dish containing ultra-pure water to rinse off any culture medium.
5. Once at testing temperature, a sterile plastic inoculation loop is used to place four *Lemna* plants of the correct stage (Figure 2) into each flask. Starting with the control, all four plants are added to the first replicate, followed by the second and third replicate. The flasks are inoculated in order of increasing contaminant concentration. This differs from other tests described here, in which the organisms are (more ideally) allocated to replicates randomly at the beginning of a test. However, the purpose of this is to minimise the use

of the plastic, disposable loops. If the *Lemna* were to be inoculated in a random manner this would require a new disposable loop for the inoculation of each plant (~80 per test).

6. Place a piece of aluminium foil on the top of each flask and remove excess foil so that the shadow in the flask is minimised. Number the foil lids of the flasks in increasing order so the flasks can be placed in the incubator in a random order each day.
7. Check that each flask contains four 3-fronded plants.
8. Use the random number sheet and the numbers on the foil lids of the flasks to place them in the incubator in random order.

Days 1-3

9. At 24 h intervals, remove all test flasks from the incubator.
10. Record the number of plants in each flask, whether there is any fungal or bacterial growth and any other observations on the test sheet.
11. Re-randomise flask positions within the incubator.

Day 4

12. Remove flasks from the incubator and arrange them in order of ascending toxicity.
13. Set up a magnification lamp, the light box, camera and tripod (Figure 3).
14. Make general observations of plant health, e.g. size and colour of fronds.

Surface area

15. Transfer all fronds within each flask into a 50 mL crystallising dish containing 50 mL of ultra-pure water, making sure that all plants are placed within the laminated scale grid with no overlapping plants (as in Figure 4).

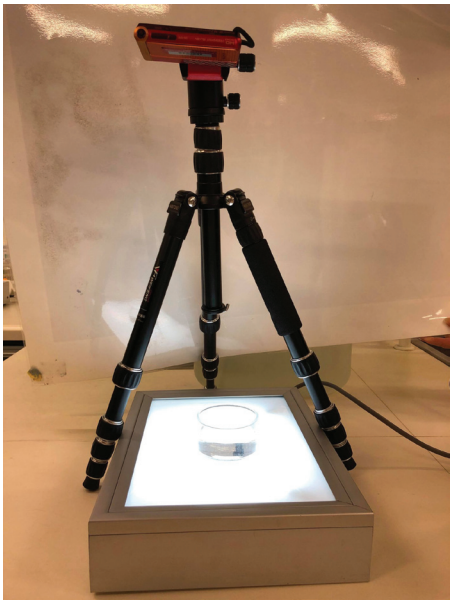

Figure 3. Set up of camera, tripod and light box for capturing surface area images.

16. The crystallising dish should then be placed on the lightbox directly under the camera with the treatment and replicate number placed next to it.
17. The camera should be zoomed to 4.8 or 4.9x zoom and the centre of the *Lemna* fronds selected on the camera touch screen so that this will be the point of focus of the photograph.
18. Selecting the centre of the *Lemna* fronds is very important, otherwise another point on the image may become the focus point and will decrease the resolution of the *Lemna* fronds for analysis.
19. Half press the shutter button on the camera so that the box highlighting the centre of the *Lemna* turns green. Then fully depress the shutter button to capture the image.
20. Repeat for each flask, in order of ascending toxicity
21. Upload camera images to a computer with quantitative image analysis software. Various open source programs can be used: ImageJ (http://rsbweb.nih.gov/ij/index.html); FIJI, a version of ImageJ (http://fiji.sc); Icy (http://icy.bioimageanalysis.org); and Matlab (https://au.mathworks.com/products/image.html). Image-Pro and Imaris are also recommended for this purpose but are not free.
22. Calculate the surface area of each image (replicate). The steps in Appendix G can be followed, but are specific to the ImageJ software.

Frond count

23. Once an image has been captured, the crystallising dish is removed from the light box and placed under the magnification lamp.
24. Count the number of plants within the dish and record it on the test sheet.
25. Using needle-nosed forceps gently remove each plant from the dish and count the fronds under magnification. A frond is counted, however small, when it is visible beyond the margin of the mother frond (Cleland and Briggs 1969). Place the frond on a tissue or white sheet of paper, and discard only once the count is complete.
26. Record the number of fronds on the test sheet.

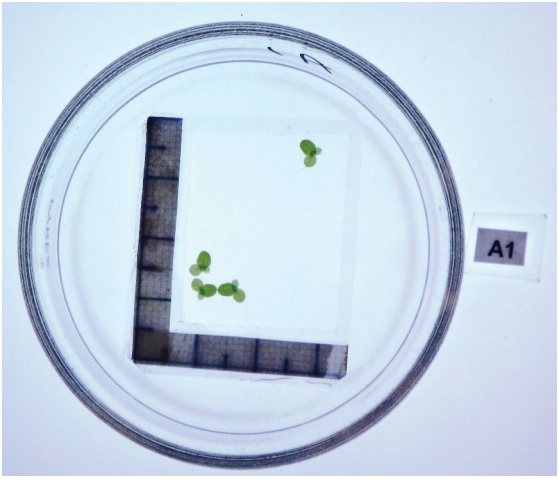

Figure 4. Setup of fronds in watch glass for capturing surface area images.

27. Pour a sub-sample from each flask in each treatment into a water parameter vial and measure the water quality parameters.

ANALYSIS OF TEST DATA

Growth rate for both surface area and frond count (K) is calculated using the formula:

Where N_t = number of fronds (or SA) at the end of the 4 d test period

N_0 = number of fronds (n_0 = 12) or SA at the start of the test period

t = length of test period in d (T=4)

The growth rate of the control treatment must be within the range 0.35 ± 0.1 cm^2/d for the SA endpoint and 0.4 ± 0.1 fronds/d for the frond count endpoint (a total of 60 fronds over 96 h) for the test to be considered valid. Growth rate data are then analysed as described in the Generic Methodology section.

MOINODAPHNIA MACLEAYI

Cladocerans or water fleas are an ideal test organism for environmental monitoring as they are known for their sensitivity to contaminants (Semaan et al. 2001). *Moinodaphnia macleayi* King (Crustacea, Cladocera) is a littoral, benthic species found in weed bed habitat on the Magela Creek floodplain (Smirnov 1983; Julli 1986; Figure 5). Under optimal environmental conditions this organism exhibits asexual reproduction and thus the cladoceran culture usually consists of genetically identical individuals, and fleas used in testing are asexually-reproducing (parthenogenetic) females. The original cladoceran culture at *eriss* was stocked with animals collected from Magela Creek, with recent cultures restocked from Sandy Billabong in November 2014.

ORGANISM CULTURE

The primary cladoceran culture is maintained in the laboratory in individual 40 mL polystyrene screw-capped vials (Cospak 12 dram clear vial, P12C). The lids have been drilled with two 2 mm diameter ventilation holes. During non-testing periods, 12 vials are kept on a Perspex tray in the incubator in the culturing laboratory at 27 ± 1°C on a 12:12 h light:dark cycle. Water and food (the alga *Chlorella* sp. and Fermented Food with Vitamins; FFV (Appendix H)) are renewed daily for each vial, and details of culture health are recorded at the time of each transfer. It is important to record when a new batch of algae, FFV or creek water is introduced to the fleas, in order to try and identify the cause of any deterioration in culture health.

A water and food renewal is performed as follows:

1. A 5 L bottle of diluent water with a dispenser is stored in the same incubator as the cladoceran culture.
2. The appropriate number of clean vials are placed on a Perspex vial tray.
3. 30 mL of FFV and a pre-determined volume of *Chlorella* sp. cells (to provide 6 x 10^6 cells/d, Appendix I) is pipetted into each vial.
4. 30 mL of diluent water is dispensed into each vial.
5. The trays containing the fresh food and water are placed on a warming tray next to the tray containing fleas to be transferred.

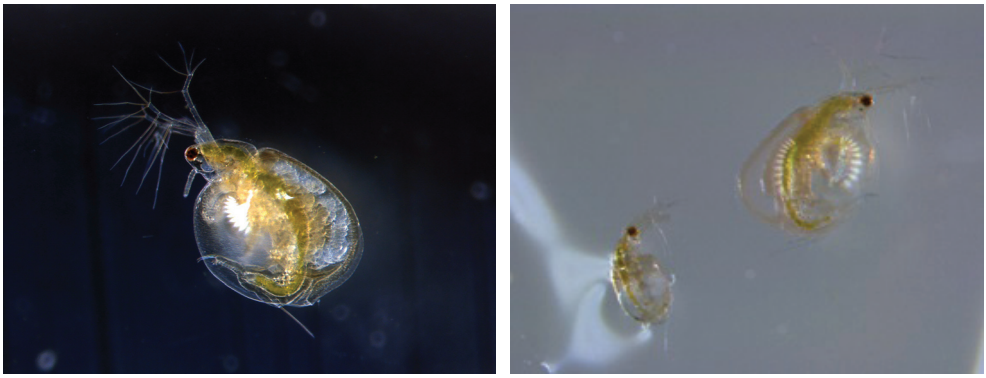

Figure 5. Left: *Moinodaphnia macleayi* female. Right: smaller male alongside larger female.

6. With the aid of a microscope, cladocerans are transferred using a wide-mouthed Pasteur pipette with a 2 mm diameter rounded tip. Pipette tips are widened by cutting off the fine end using a diamond cutting tool/pen, then smoothed by sanding and flaming the cut edge over a Bunsen burner. Glass pipettes are preferably used as they enable the handler to closely observe the cladoceran while it is in the pipette. However, if it is not possible to prepare glass pipettes as described above, a plastic pipette with the end snipped off would suffice. Care should be taken to ensure that the snipped end is smooth so that it will not damage the cladocerans during transfer.

At *eriss*, the cladoceran culture is maintained in only one type of water, filtered MCW. *M. macleayi* do not survive long term in SSW.

The cladoceran 'culture cycle' follows the generation of three broods. This generally takes 6 d:

Day 1: A culture tray is started with 12 brood-2 neonates (1 neonate per vial).

Day 2: Neonates are transferred to new water and food.

Day 3: Neonates will have developed into adult females and had their first brood (brood-1 neonates can be discarded). The 12 adults are transferred individually to new water and food.

Day 4: Females will have had their second brood. Adults are transferred individually to new water and food. A new tray of neonates is prepared from the brood-2 neonates (using 34 different broods). Remaining neonates are used to stock a back-up culture maintained in a bowl.

Day 5: Females are transferred until they have their third brood (this provides an indication of health and is run parallel with the test, which runs until the third brood has been produced). The tray of new neonates (started on Day 4) is also transferred to new water and food.

Day 6: Adult females should have produced brood 3. Both the females and brood-3 neonates can be discarded. The tray of new neonates (started on Day 4) are now adult females which should have had their first brood. The females are transferred to new water and food to continue the cycle and the brood-1 neonates can be discarded.

PRINCIPLE OF THE TEST

Female cladoceran neonates that are <6 h old at the beginning of the test, are exposed to a range of contaminant concentrations or effluent dilutions under static renewal conditions. The females are transferred daily to fresh solutions of the same concentration. Each day, observations are made on the survival of each female, the number of neonates produced and neonate survival. Each female must be accounted for as alive, dead or missing. The test is terminated when three broods have been produced by each surviving control female (normally over a 56 d period). The method is based on the *Ceriodaphnia* Survival and Reproduction Test developed by the USEPA (Horning and Weber 1985) and was initially developed using a local species by McBride et al. (1991).

TEST SOLUTIONS

Treatments are prepared in bulk 3 L volumes at the start of the test (or the day prior if equilibration is required) and stored in 5 L screw-capped polyethylene bottles in a refrigerator. Test solutions are prepared by either diluting a stock solution or effluent with diluent water. If the pH of the test solution is not within the test organism's range of tolerance, the pH can be adjusting using H_2SO_4 or NaOH.

TEST PREPARATION

Prior to starting a toxicity test, the number of neonates used to restart the incubator stock culture should be increased to 36. These will require the same maintenance as general cultures but should be carefully observed in order to determine when they will have their second brood. Neonates should be <6 h old at the test start. This can be difficult to predict and can occur at inconvenient times. If the second brood is likely to arrive at an inconvenient time, a further 24 to 36 culture vials can be set up using neonates from the third brood of the original 12 culture fleas. Although it is preferable that a test is started with offspring from the second brood, this provides another option for test start time. It is important to ensure that there is sufficient food (algae and FFV) prepared for a toxicity test.

ACCLIMATION

Cladocerans cannot be transferred from one water source to another of different water chemistry without causing osmotic shock and possibly death. Instead, the water fleas must be introduced over several days to the new water source by adding increasing amounts of the new water to the original water.

For example, if running a test using a different diluent water to that used for culturing, gradually acclimate the culture as follows:

Day 1: Set up an extra culture in the original water.

Day 2: Feed and transfer into 100% original water.

Day 3: Feed and transfer into 75% original water and 25% new water.

Day 4: Feed and transfer into 50% original water and 50% new water.

Day 5: Feed and transfer into 25% original water and 75% new water.

Day 6: Feed and transfer into 100% new water.

Throughout this acclimation, carefully observe the animals for signs of stress such as the presence of males or slow development. Males are typically much smaller than females, and are more elongated (less round) than females (Figure 5). If an individual is smaller than it should be (adult females are ~1 mm diameter) and is 4 d-old without having produced neonates, it is likely to be a male and should be discarded. If the culture seems to be of sub-optimal health, the acclimation process should be stopped, or slowed to allow the animals to recover and adjust to the new conditions.

COMMENCING A TOXICITY TEST

1. The start time of the test will depend on when adult females are due to have their second brood. This can be calculated by adding 28 h onto the time the first brood arrived. Another indicator is that females will have full brood pouches and their neonates' eyes will be showing as black dots.

2. Once enough females have produced the second brood, the broods can be pooled into a large watch glass and returned to 27°C until the test is started.

3. Preparation for the test (e.g. preparing test solutions) should be carried out so that the test can be started as soon as there are sufficient brood-2 neonates. This ensures neonates are as young as possible when the test is started (neonates should be <6 h old).

TEST PROCEDURE

Day 0

1. Label a Perspex tray for each treatment (A, B, C, D, etc), set up 10 x 40 mL vials on each tray and label vial lids appropriately (e.g. A1, A2, A3).
2. To each test vial add 30 µL FFV and the required volume of *Chlorella* sp. algae to provide a total of 6×10^6 cells. Then immediately dispense 30 mL aliquots of each test concentration into the appropriate vials and allow at least 1 h to equilibrate to incubator temperature (27 ± 1°C).
3. At the time of dispensing each treatment, also dispense and cap a sample of each test solution (50 to100 mL with food and algae added at the correct density) into appropriately labelled 150 mL containers for the measurement of fresh water quality parameters (pH, EC and DO).
4. Remove trays with vials of test solutions from the incubator and place on a warming plate.
5. Using a stereo microscope, place the watch glass of neonates on the stage and with a glass Pasteur pipette with tip <1 mm, pick out one test neonate from the watch glass, and drop gently into the first control replicate without touching the pipette into the test solution.
6. Repeat for the first vial of each test concentration, working up in concentration and ending with the highest concentration.
7. Discard the pipette and obtain a clean one if the test solution was touched at any point or if the pipette has been dropped or come in contact with a contaminated surface.
8. Repeat steps 5 and 6 for the other vials until all 10 vials for each concentration contain one neonate. If it is likely this process will take one person longer than half an hour to complete, it is recommended that two people distribute test neonates simultaneously.
9. Under the microscope, cross-check each vial to ensure there is one neonate in each vial.
10. When one neonate has been distributed into each of the vials, cover vials with labelled plastic lids with air holes and randomise their order on the tray.
11. Place the trays in the incubator in their random order for that day. Completion of this stage constitutes the start of the test (time = 0 h) and is recorded on the information sheet in the test folder.
12. Measure water quality parameters (pH, EC and DO).

Day 1

13. Dispense test solutions into appropriately labelled vials (which already contain FFV and algal food). Also dispense a 50 to 100 mL sample of each solution and food supplements and measure pH, EC and DO, as in step 3.
14. Place a cover over the top of the entire tray to prevent any airborne dust or contaminants from entering the vials and allow dispensed solution to equilibrate to 27 ± 1°C in an incubator.
15. 24 h after the commencement of the test, remove trays from incubator and sort the test vials into numerical order for each concentration group.

16. For each concentration group, observe and transfer each live cladoceran into the appropriate replacement vial of new water. Place the labelled lids on vials immediately.
17. Record whether the cladoceran is alive, dead or missing; whether eyed-young are seen in the brood chamber; if there are any neonates present, and if any of them are dead. A cladoceran can be considered dead if there is no movement after being gently brushed with the pipette.
18. Record any other observations on health that suggest that the cladocerans are not developing normally, such as inability to moult, small size, etc.
19. To avoid observer bias, each day select a different concentration group to observe first.
20. Keep the old water aside to count neonates later (usually present from d 2).
21. Use a clean pipette if observing a lower concentration solution than the previous one.
22. When test water fleas from all concentration groups have been transferred into new water, place vials into the random order for that day, and place trays into the incubator in the random order for that day.
23. For each of the old vials containing the previous day's test solution, count and record the number of neonates, firstly the total number (alive and dead) and then secondly, the number of dead neonates. The dead neonates will lie on the bottom of the vial, whereas the live neonates will swim. Pipette and remove each neonate into a separate container as it is counted.
24. After the neonate count is complete, a sub-sample of the old test solution for each concentration is collected, representing the 24 h-old water parameter measurement.
25. Measure and record the water quality parameters (pH, EC, DO) of the 24 h-old test solution samples.

Day 2 to 5

26. Repeat steps 13 to 25 (i.e. at 24 h intervals, transfer live test water fleas to fresh solution and record observations, count and record neonate number, and record the water quality parameters for the appropriate day).

Day 5 to 6

27. Count and record observations of water fleas and the number of neonates produced (total and number dead), but if the test is due to finish that day, do not dispense or transfer test water fleas to fresh test solution.
28. Take sub-samples of old water for chemical analysis if necessary and measure and record the water quality parameters.

The test is complete when three broods have been produced by 80% or greater of control water fleas and have been counted. This usually occurs by d 5 or 6, but if not, the test should be continued to d 7. For analyses to be performed, ≥80% of the control cladocerans must be alive, and must have produced three broods, averaging ≥30 neonates at the end of the test period.

ANALYSIS OF TEST DATA

The number of neonates across the three broods is summed for each replicate. A mean total number of neonates is calculated for each treatment. The reproduction of each treatment is presented as a function of the control response and plotted against measured toxicant concentrations or effluent dilutions. Statistical testing should not proceed if fewer than four treatments (including the control) remain. Reproductive data are then analysed as described in the Generic Methodology section.

HYDRA VIRIDISSIMA

Hydra viridissima Pallas (Cnidaria, Hydrozoa) is referred to as green *Hydra* because of its green colouration due to the presence of a symbiotic green alga in the gastrodermal cells. Although the precise distribution of this species has not been mapped, it has been found in a variety of aquatic habitats in northern Australia. *Hydra* were originally collected from Magela Creek in 1993 and have been cultured continuously at eriss.

To be selected for a test, a hydroid must have only one developing bud (Figure 6). The bud must not be fully developed; tentacles should be present only as bumps and the bud must not be ready to detach from the body stalk. Asexual budding is a characteristic behaviour of Hydra in optimal environmental conditions. *Hydra* selected for testing must be free of overt disease and gross morphological deformity (i.e. show no signs of tentacles clubbing or contracting).

ORGANISM CULTURE

The primary culture of green *Hydra* (*Hydra viridissima*) is cultured in the laboratory in bubble-aerated filtered creek water, synthetic water or filtered Darwin tap water, held in 2 L flat-based, shallow glass dishes (hereafter referred to as 'culture dishes'). The dishes are covered with a clear section of Perspex that lets light through while protecting the culture from airborne dust and contaminants. The Perspex sheet has a 5 mm hole drilled in the centre through which an airline with an air stone is fitted. The air stone is fully submerged in the culture water and provides gentle aeration. The water movement caused by the gentle aeration causes most *Hydra* to attach to the dish surface via the basal disc, which makes water changes easier. The primary culture is kept in a temperature-controlled incubator (27 ± 1°C, 12:12 h light:dark cycle) and a secondary culture dish is maintained on a bench at 28°C. A third back-up culture is kept in a tank that houses fish brood stock. This culture that attaches to the inner sides of the tank is not actively maintained except for occasional feeding with excess brine shrimp.

Feeding

Primary and secondary *Hydra* cultures are fed every second day and observations on health and density are made prior to feeding. One week prior to commencing a test, *Hydra* are fed daily to optimise budding. *Hydra* are fed with 2 d-old brine shrimp nauplii (Appendix J) and are left for 24 h prior to cleaning.

Figure 6. Left: *Hydra viridissima*. Right: Correct stage of *Hydra* for test start (with developing bud).

Cleaning

At the end of the feeding period, uneaten brine shrimp and regurgitated food is removed by performing a scrub clean. Before performing this procedure, hands should be washed with soap and rinsed well, with a final rinse in RO water.

1. Pour dirty water from the culture dish into a glass cleaning bowl (most *Hydra* should remain attached to the surface of the culture dish, but those that are floating and are tipped out into the cleaning bowl can be pipetted back to the clean culture dish later if need be).
2. Add ~50 mL of diluent or culture water, swirl around the dish to rinse the base and then tip into another cleaning bowl. If the culture density appears to be too high and needs to be reduced, the *Hydra* that detach or are lost during this step (and step 1) can be directly discarded down the drain or added to the third back-up culture in the fish tank. Where *H. viridissima* are being used by laboratories located in southern parts of Australia, the *Hydra* will need to be euthanased before discarding. This can be done instantly by adding concentrated Decon Neutracon solution to the bowl or tray containing the *Hydra* (to ~1% strength). The *Hydra* can then be discarded.
3. Add another ~50 mL of diluent water to the culture dish and dislodge *Hydra* into the water by using a finger to gently remove them from the surface of the dish.
4. The water containing the dislodged *Hydra* is swirled around and poured into another cleaning bowl.
5. The culture dish is then cleaned by adding RO water and rubbing the surface clean with fingertips. This water can be discarded and the surface rubbed dry with a lint-free tissue. Culture dishes are also periodically cleaned in an analytical-grade dishwasher.
6. The detached *Hydra* can be allowed to settle onto the surface of the cleaning bowl before tipping off waste water, adding new water and dislodging the *Hydra* into the clean water to be poured back into the clean culture dish. Alternatively, floating *Hydra* can be swirled into the centre of the cleaning bowl and transferred (with a Pasteur pipette) into ~1.5 L of new culture water.
7. For optimal health, *Hydra* should be maintained at a density scale of 2 to 3 (Figure 7).

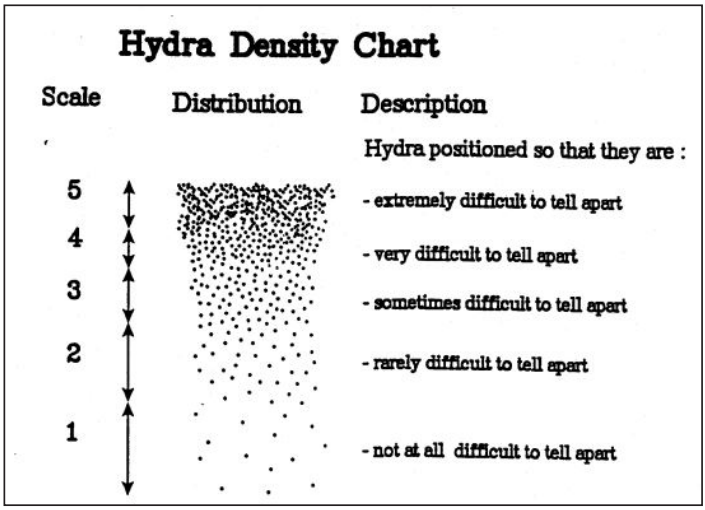

Figure 7. Density scale for assessing *Hydra* density.

8. If white egg masses (on the body stalk) are observed in the culture, this indicates the culture is unhealthy and should not be used for testing. Culture conditions should be checked. Conditions may be improved by reducing culture density and ensuring cultures are being fed good quality brine shrimp and cleaned thoroughly. Lighting and temperature should also be checked.

PRINCIPLE OF THE TEST

Asexually reproducing (budding) *Hydra* are exposed to a range of contaminant concentrations or effluent dilutions for 96 h. Observations of any changes to the *Hydra* population (i.e. changes in the number of intact hydroids, where one hydroid equals one animal plus any attached buds) are recorded at 24 h intervals. The method is based on the *Hydra* population growth test described by Hyne et al. (1996).

TEST SOLUTIONS

Treatments are prepared in bulk 2 L volumes at the start of the test (or the day prior if equilibration is required) and stored in 2 L screw-capped polyethylene bottles in the refrigerator. Test solutions are prepared through dilutions of either a stock solution or effluent with diluent water and then pH adjusting the solution if necessary, using H_2SO_4 or NaOH.

TEST PREPARATION

1. Set up three Petri dishes for each treatment on flat Perspex trays (due to the low variability in growth rates, two replicates may be used if necessary, to allow for more treatments to be included in a test). Each Petri dish should be labelled with treatment and replicate number. The Perspex trays can also be permanently labelled with eight different numbered positions that aid with daily randomisation of the position of the replicate on the tray.
2. Set aside an extra 23 Petri dishes for each treatment to hold water for chemistry samples.
3. Arrange 3 x 40 mL labelled vials for each treatment on a Perspex tray to be used for water changes on days 2, 3 and 4.

TEST PROCEDURE

Day 0

1. Prepare test solutions (or prepare the day prior if equilibration is necessary) and leave at room temperature.
2. Dispense 30 mL aliquots of each test concentration into three labelled replicate Petri dishes (i.e. 3 x 30 mL for each test solution), and also into additional Petri dishes for each treatment that is used for chemistry. Place trays of dishes into the incubator for at least 1 h to allow test solutions to reach 27°C.
3. Dispense 60 mL of each test concentration into labelled 120 mL plastic containers for water parameter analysis and place in incubator.
4. Isolate enough suitable *Hydra* (30 per treatment) into diluent water across three Petri dishes and place in test incubator to equilibrate to test temperature.
5. Water parameters of the test solutions should ideally be checked before the *Hydra* are transferred to test solutions. Measure and record EC, pH and DO of the water parameter samples starting with the control and working up in order of ascending treatments.

6. Remove trays with Petri dishes of test solution from the incubator and place them on warming trays near a stereo microscope.

7. Remove Petri dishes containing *Hydra* from the incubator and place on a warming tray.

8. Using a Pasteur pipette, select *Hydra* individually from the isolated stock and place into control replicate 1, working up in concentration until all replicate 1 dishes have ten Hydra. This should be done without letting the pipette tip to contact the test solutions in the Petri dishes.

9. Discard the used pipette and select a new one if at any time it comes in contact with any of the test concentrations.

10. Repeat steps 8 to 9 until all test dishes for replicates 2 and 3 contain 10 *Hydra*.

11. Observe each dish under the microscope to ensure that there are ten *Hydra* in each dish, and replace any *Hydra* that are damaged in any way (e.g. all buds must be attached). If damaged, replace immediately with suitable test *Hydra* using a new pipette. It is preferable that this check is not performed by the same person who originally pipetted the *Hydra*. If more than one person distributed the test *Hydra*, it is good practice to check the treatments that were started by the other person and vice versa.

12. Place the labelled lids on the dishes and position dishes in randomised positions on the trays for that day.

13. Place trays in the incubator and record the test start time.

Day 1

14. Dispense 60 mL of each treatment into labelled 120 mL vials for water parameter measurement. Place vials in incubator to warm to test temperature.

15. Dispense 3 x 35 mL of each test solution into labelled 40 mL vials and place trays of vials in the incubator to equilibrate to test temperature.

16. Measure and record water parameters on new test solutions for all treatments and adjust test waters if required.

17. 20 h after the commencement of the test, remove the trays from the incubator, arrange the Petri dishes into replicate groups, observe *Hydra* under the microscope and record as d 1 observations. Water movement will cause temporary tentacle contraction, therefore, allow the water to settle before recording observations. If possible, to avoid observer bias, have two people perform observations and feeding with each person working with a different set of replicates each day.

18. After recording observations for a particular dish, feed each *Hydra* in the dish individually. Each *Hydra* is fed at least 3 or 4 live brine shrimp nauplii using a Pasteur pipette (Appendix J). *Hydra* are left to feed for 4 h and should be fed in ascending treatment order to prevent contamination.

19. Place Petri dishes onto trays in the random order for the day and return trays to the incubator.

20. 24 h after the commencement of the test, the trays of Petri dishes are placed on warming

trays near the microscope. Petri dishes are not re-ordered but are left in their randomised positions on the tray. The solutions are renewed as follows:

a) The test solution is swirled around the Petri dish to dislodge any uneaten brine shrimp and regurgitated food. If brine shrimp are difficult to remove they may need to be gently puffed with solution using a glass Pasteur pipette.

b) The solution is then tipped carefully into a waste Petri dish and checked for any dislodged *Hydra*, returning them to the test Petri dish. The waste water is then transferred to the corresponding water parameter vial.

c) An aliquot of the test solution (5 mL) is immediately added to the Petri dish to cover the *Hydra*, swirled, and the solution tipped into the waste Petri dish.

d) The remaining fresh solution (30 mL) is immediately added to the test dish.

e) Any *Hydra* that are dislodged into the waste dish are carefully picked up with minimal waste water using a clean pipette and returned to the test dish.

f) Any remaining brine shrimp, or other debris, in the test dish are removed by pipette, while minimising removal of test solution.

g) The cleaning dish is checked again for *Hydra*, with any found being returned to the test dish.

h) The solution in the cleaning dish can be discarded to a waste beaker.

i) Repeat steps a) to h) for each of the three sets of replicates in ascending treatment order. If necessary, use a new pipette and cleaning dish if a dish of lower chemical concentration needs to be cleaned after a higher concentration.

21. Once test solutions have been renewed for all dishes, return trays of Petri dishes to the incubator.

22. Measure the physical water quality parameters (i.e. pH, EC, DO) of the 24 h-old water.

Day 2–3

23. Steps 15 to 22 are repeated (i.e. at 24 h intervals, measure and adjust test water if necessary, count and record observations for the appropriate day, feed test organisms, and clean and renew test solutions).

Day 4

24. Count and record final observations for each test dish 96 h after the start of the test. Do not feed *Hydra* and do not renew test solutions.

25. Old test solutions can be poured into vials to measure and record Day 4 water quality parameters or sub-sample for chemistry if required.

26. Test *Hydra* are euthanased by adding ~0.1 mL of Decon Neutracon detergent to each Petri dish and scrubbing the base of the dish before discarding *Hydra* down the drain.

ANALYSIS OF TEST DATA

The population growth rate of each replicate is calculated based on the number of *Hydra* at the start and the end of the test using the following equation:

$$\frac{\ln(Day\ 4)-\ln(Day\ 0)}{4}$$

The growth rates of the replicates are averaged to produce a growth rate for each treatment. More than 30 healthy hydroids must be present in each control dish (equivalent to a growth rate of 0.27) at the end of the test period for the test to be considered valid and for analyses to proceed. Population growth rate data are then analysed as described in the Generic Methodology section.

AMERIANNA CUMINGI

The test species, *A. cumingi*, is a pulmonate snail of the Planorbidae family. It is dark in colour and grows up to 16.5 mm in length (Figure 8, Jones 1992). *A. cumingi* is characterised by its rounded shoulders and a truncated spire (Jones 1992). The advantages of using this species in toxicity testing are that it inhabits waterways in the Alligator Rivers Region and that it can be easily transported and cultured in the laboratory. These pulmonate snails are hermaphroditic (have both male and female sexual characteristics), which means separate males and females of this species do not need to be sourced for reproductive research (Suggit 1992). *A. cumingi* also produces a large numbers of eggs (Ravera 1991), which are within round egg masses that are easy to observe (Figure 8; Suggit 1992). *Amerianna* species are also particularly sensitive to toxicants as they do not possess an operculum and cannot seal off their body from the environment (Suggit 1992).

ORGANISM CULTURE

Snails used in testing are either obtained from flow-through tank cultures maintained in filtered Darwin tap water at 28 ± 2°C or from outdoor large-scale tubs. The indoor tanks (~200 L) have suspended cool white lighting providing a 12:12 h light:dark cycle. The outdoor tubs are subject to natural light and diurnal cycles, and are not temperature-controlled. Freshwater pulmonate snails are grazing herbivores, with their diet in the wild consisting of living and decaying plant material such as epiphytes and/or detritus (Humphrey et al. 1995). Cultured snails are fed the outer green leaves of organic iceberg or cos lettuce (after rinsing in deionised water) and sprinkles of fish flake (Sera San, Heinsberg, Germany) daily. If the snails are to be cultured in indoor tanks, the broodstock are collected from the Jabiru Field Station as required, and it is the offspring produced from these snails that are used in testing. If snails used in testing are sourced from the outdoor large-scale tubs they should be selected and transported to the laboratory 3 to 5 d prior to the test start date to minimise the stress associated with transport during the test.

Feeding

Snail cultures are fed a diet of the green outer leaves of organic lettuce (either cos or iceberg) and flake fish food (Sera San) every day. Lettuce leaves are washed thoroughly with filtered tap water to remove soil and other organisms. The lettuce is then placed in the tanks and anchored to the base

Figure 8. Left: Adult *Amerianna cumingi*. Right: *A. cumingi* egg mass.

using Perspex tubes. This enables the snails to access the lettuce more readily. A small amount of lettuce can also be left on the surface of the water. Add enough lettuce so that only a small amount remains after 24 h. Any decaying lettuce in the tanks should be removed.

Approximately 20 to 50 g of flake food (depending on snail density) is added to the tanks to provide additional nutrition. Feed no more than the snails can consume in 3 h. Overfeeding (of flake food in particular) will result in water fouling and may provide more favourable conditions for pest species such as ostracods or flatworms. Generally, at low densities, these do not pose any problems to snail cultures, but should be observed to ensure that they do not adversely affect snail populations.

CLEANING

Flow rate into the aquaria, aeration, and snail health should be checked daily and adjustments made as necessary. Snail tanks should be siphoned to prevent build-up of wastes that may affect stock health and reproduction. It has been found that monthly siphon cleans are sufficient to maintain good water quality and infrequent enough to maintain good snail density (i.e. young snails can be accidentally removed during siphoning).

The following water quality parameters should also be measured and recorded on a weekly basis; chlorine and TAN (which can be measured using colorimetric kits), DO, EC, pH and temperature.

PRINCIPLE OF THE TEST

A standard number of healthy snails are exposed to a range of toxicant concentrations or effluent dilutions for 96 h under controlled conditions. The number of eggs produced by the snails is counted at the conclusion of the test. The reproductive performance of the snails is assessed by comparing egg production of snails exposed to various concentrations of a chemical or water sample to that of control (non-exposed) snails. Egg production is used as an endpoint as earlier studies have shown it to be the most reliable and sensitive endpoint for toxicity testing with *A. cumingi* (Lewis, 1992; Burrows-Ellis 1994).

TEST SOLUTIONS

1. Each treatment requires 22 L (one jerry can) of test solution for the 96 h test. A sufficient volume of diluent water required to prepare test solutions should be removed from the refrigerator and left overnight in the same room, or at the same temperature (~28°C) as the snail culture.

2. Prepare test solutions (the entire 22 L volume for each treatment) preferably the day before the test start. After preparation, each treatment is poured into a jerry can for storage over the 96 h test.

TEST PREPARATION

3. Feed the snail culture the afternoon prior to toxicity testing. This attracts the snails to the leaves, which can then be lifted out on the day of the test start.

4. Set up labelled 2 L silanised beakers for each treatment (three replicates per treatment).

5. Set up labelled Perspex tubes for each treatment on a large Perspex tray, with one end of the tube enclosed by a PVC clip and mesh (~0.5 mm mesh size, Figure 9). The other end is left open until the snails and lettuce discs have been added.

6. Place a 40 L Nally bin on a trolley (preferably at waist height) and fill it with the water

Figure 9. Tube enclosure for a pair of *Amerianna cumingi* snails.

that will be used as the test diluent. Cover the container to prevent evaporation and contamination, and submerge an aquarium heater, to warm the water to test temperature overnight. An aerator can be set up but the water will not require aeration until snail tubes are transferred. Check water is test temperature before transferring snails to the Nally bin.

7. Ensure there is sufficient lettuce to conduct the test.

Isolation of test snails

1. Ensure your hands and arms are clean prior to handling snails.
2. Snails selected for testing must be between 10 and 13 mm long from the tip of the anterior end of the shell running down the middle of the shell to the shoulder of the posterior end (do not include the spire when measuring length; Figure 10). Vernier calipers are used to measure the snails.

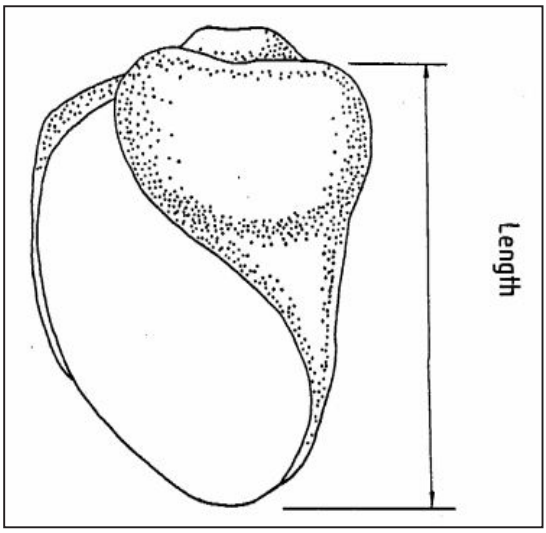

Figure 10. Ventral view of *A. cumingi* showing length measurement (Jones 1992).

3. Snails should be free of scaling or shell damage. Snails should not be used in testing if the body cavity is not firmly attached to the shell and appears to be pulling back from the edge of the shell, as this may suggest that the snail is not of optimal health.

4. When collecting test snails they should be carefully removed from their substrate (tank glass or lettuce) so as not to damage them. Applying a small amount of pressure to the posterior end of the muscular foot using the index finger, will encourage the snail to release its grip on a substrate with minimal stress on the individual.

5. Caution should be taken to ensure that flatworms (if present in the culture tank) are not accidentally transferred along with the snails into the test containers. Any egg masses that may be present on the shells of snails must also be gently wiped off before the snail can be used in testing.

6. If possible, have two people isolating the test snails; one of whom isolates, measures and places the snail pairs in tubes, the other distributing lettuce discs and placing the mesh and clip on the tube and submerging tube into the Nally bin of diluent water to be held for the test. The amount of time the snails are held out of water should be kept to a minimum.

TEST PROCEDURE

Day 0

1. For each treatment, pour 1.75 L of test solution into each of three 2 L beakers, cover with cling wrap and number each replicate (e.g. 1 to 24), going up in increasing order through the treatments, by labelling the cling wrap cover with permanent marker. This provides a number that can be used for randomising the position of the beakers in the incubator. Then equilibrate beakers to 30 ± 1°C in an incubator. Fill 5 L bottles with the test solutions for each treatment for Day 1 and leave them in another incubator with the lights turned off until the next day.

2. Dispense test solutions into water chemistry bottles and also into containers for dissolved oxygen, electrical conductivity and pH measurement.

3. While the test solutions are warming, lettuce discs (2 cm^2) should be cut from fresh, crisp cos or iceberg lettuce, rinsed in Milli-Q® water and stored in a beaker in the refrigerator.

4. Begin aerating the acclimation tub and ensure the water is 30 ± 1°C before starting to isolate test snails.

5. Isolate the test snails from the culture tank, measuring each snail to ensure it is between 10 and 13 mm in length. As snails are picked from the tank, a pair should be carefully placed in each tube along with two discs of lettuce and the tube sealed off with a piece of mesh and PVC clip. (This is done as quickly as possible out of water.) Tubes are permanently pre-labelled with treatment and replicate number and are selected randomly to avoid allocating differences in the snails' health or size to certain treatments.

6. Once all tubes contain a pair of snails, the acclimation tub containing the tubes can be moved to where the snails will be added to the treatment beakers.

7. All test beakers are removed from the incubator and set up on a bench.

8. Snail tubes are taken from the acclimation tub and placed in the appropriate replicate (matching up the label on the tube with the correct beaker) until each beaker contains six snail tubes.

9. The beakers are re-covered with cling wrap and then randomly positioned in the incubator using a random number sheet and the numbers labelled on the cling wrap of each beaker.

10. Aeration is provided from aquarium air pumps to each beaker through vinyl airline tubing and long-tip glass pipettes which are inserted through the cling wrap into the test solutions.

11. Record the test start time, as daily renewals and feeding will need to be done at this time over the next 96 h.

Day 1

12. Cut lettuce discs and keep refrigerated until required.

13. At the test start time, remove Treatment A replicates from the incubator and fold back the cling wrap covering the beakers.

14. Pour approximately 30 mL of waste water from each of the replicates into a polycarbonate vial to measure DO, EC and pH (these can be measured at a later stage). Use 5 mL of this pooled sample to measure TAN with a colorimetric test kit. This should be done immediately to ensure accurate analysis. The concentration of TAN in the 24 h-old water should be ≤1.0 mg/L for the control and all treatments (unless TAN is a known elevated contaminant in the test water).

15. Start with one beaker, removing one snail tube at a time. Remove the clip and mesh from one end of the tube and rinse these in a beaker of Milli-Q® water. Count and record the number of egg masses on the tube, check there are two snails still alive and add two lettuce discs before closing the tube. The tube containing the snails should be placed back into the treatment beaker as quickly as possible. If one or both snails in a tube have died, this is recorded and the tube is removed from the replicate.

16. Repeat this for all six tubes in each of the three replicates for Treatment A.

17. All six tubes are then removed from each beaker, the old water is discarded and each beaker is filled with approximately 1.75 L of new test solution before the snail tubes are placed back in the beaker. The beaker is not rinsed prior to refilling. Approximately 60 mL of this new water needs to be set aside in a polycarbonate 120 mL vial for measuring DO, pH and EC.

18. Treatment A replicates are then placed back into the incubator and the replicates for treatment B are removed from the incubator.

19. This procedure is repeated for all the remaining treatments working in ascending order of concentration.

20. Fill 5 L bottles for each treatment with test solution and leave in the incubator for the following day.

Day 2

21. Repeat steps 12 to 20.

Day 3

22. Repeat steps 12 to 19.

Day 4

Because this is the last day of the test, the snails do not require feeding or water exchange.

23. Remove Treatment A replicates from the incubator. Uncover beakers and handle one tube at a time, removing mesh and clips from both ends. Remove the snails (with care to prevent damaging any egg masses) and discard them into a waste container with water.
24. Count and record the number of egg masses on the tubes and stack the tubes in a Nally bin of water (to prevent the egg masses from drying out).
25. Repeat this for the six tubes in each of the replicates for Treatment A.
26. A sample of water from each replicate should be kept for measuring DO, EC, pH and TAN as described for Day 1.
27. Repeat the above steps for the remaining treatments.
28. The test snails are euthanased by placing the waste container in a freezer.
29. Each tube is then examined under a dissecting microscope (10x magnification) and the number of eggs in each egg mass is counted and recorded for each pair of snails. An alternative method of counting egg numbers using digital photography has also been developed and can be found in Appendix K.

ANALYSIS OF TEST DATA

The number of eggs in each egg mass laid by each pair of snails is added to generate the total number of eggs produced by each pair. As each replicate has six pairs of snails, the number of eggs per pair is averaged across each replicate. The mean, SD and SE are calculated for each treatment. The %CV is calculated for the control treatment as an indicator of the within-treatment variability for the experiment. Statistical analyses are undertaken on the mean number of eggs per pair for each replicate. Data are considered valid if 80% or more of the control snails are alive, the %CV for the control is ≤20%, and eggs numbers of the control are between 100 and 250 eggs per pair. This range is based on control data from 16 tests presented in Houston et al. (2007), with the mean being 150 eggs per pair. Reproductive data are then analysed as described in the Generic Methodology section.

VELESUNIO SPP.

Several species of the genus *Velesunio* (Hyriidae family) are found throughout tropical northern Australia, the most common being *V. angasi*. A culture of mussels is not maintained in the laboratory. When required for testing, adult *Velesunio* spp. (Figure 11) are sourced from Magela Creek (latitude 12° 40' 28" S, longitude 132° 55' 52" E), or Gulungul Creek (latitude 12° 39' 21" S, longitude 132° 52' 42" E) and *V. angasi* from Sandy Billabong (latitude 12° 54' 4" S, longitude 132° 46' 38"E), and Mudginberri Billabong (latitude 12° 35' 33" S, longitude 132° 52' 33" E) within the Alligator Rivers Region, or Lake Bennett, a semi-urbanised lake located 80 km south-east of Darwin (latitude 12° 57' 39" S, longitude 131° 09' 59" E).

Adult mussels (≥4 cm) are transported live, by road to the laboratory at ambient temperature (28 to 34°C) within 4 h of collection, in aerated plastic 20 L drums. The drums contain ~15 L of sampling site water and ~15 cm of sand, collected from Magela Creek, which has been pre-sterilised by oven-drying at 60°C for 23 d.

ORGANISM CULTURE

Once adult mussels have released glochidia, the adults do not need to be maintained as a culture. However, if they need to be held in aquaria for extended periods until, for example, their release back to the collection site, mussels from each site should be kept in separate tanks and not mixed together. Up to 25 mussels can be placed in an aerated 144 L glass aquarium tank containing ~10 cm of sand, and flow-through FDTW or water collected from the site. Mortality of mussels was observed to increase if stocking density exceeded this number. The sand used in tanks and for transport is sourced from Magela Creek or Sandy Billabong and sterilised (as described above) to reduce the chance of parasitic infections. The tank should not be fitted with a bio-filter as this removes food for the mussels. The tanks should be cleaned periodically (approximately every 2 weeks) by scrubbing the algal build-up from the sides, and removing waste matter from the sand by siphoning just above the sand layer. The tank should be checked daily for dead mussels, and any placed in a freezer for disposal.

Figure 11. ***Velesunio*** **sp.**

FEEDING

Mussels are fed algae (*Chlorella* sp.) cultured in the laboratory. This needs to be prepared at least one week before mussels are collected. A conical-based 75 L tank with a central stand pipe (similar to that used for some rotifer cultures) is used to grow continuous bulk batches of algae. This culture can either be maintained indoors in a temperature-controlled room with sufficient lighting, or if outdoor temperatures are suitable, it can be in an undercover area outside, covered with a clear Perspex lid. The tank is filled with FDTW supplemented with either algal culture medium (MBL) or fish culture water seeded with 2 to 3 flasks of 100 mL algal culture, and aerated vigorously and continuously with 1 or 2 airstones. Four litres of algae from the culture tank is fed to each aquarium of mussels each day. Mussels can also be fed algae directly from the flask starter cultures; ~100 mL per tank, every 1 to 2 d, if there is insufficient algae available in the bulk tank culture. Algal discs or wafers (Hikari) can also be used if algal suspension is not available. The volume removed from the algal culture tank for feeding is replaced with filtered tap water.

PRINCIPLE OF THE TEST

Glochidia (larval stage of the mussel) are exposed to a host fish species (*Mogurnda mogurnda*), where they attach to the gills and fins of the fish to develop into juveniles (Figure 12). Following release from the fish, the juveniles are exposed to a range of toxicant concentrations for 14 days under controlled conditions. Growth (based on shell length) is measured at Days 8 and 14 and the growth rate is compared to that of control (non-exposed) mussels to obtain an EC10 and EC50. Earlier studies (Newton et al. 2003; Bringolf et al. 2007; Wang et al. 2007; 2011) have shown that, because of their sensitivity, freshwater mussels are ideal for assessing the toxicity of contaminants. These studies have also demonstrated that juvenile growth rate is the most reliable and sensitive endpoint for toxicity testing. It is also recommended by the ASTM (2006).

TEST SOLUTIONS

1. A stock solution of the required chemical is prepared.

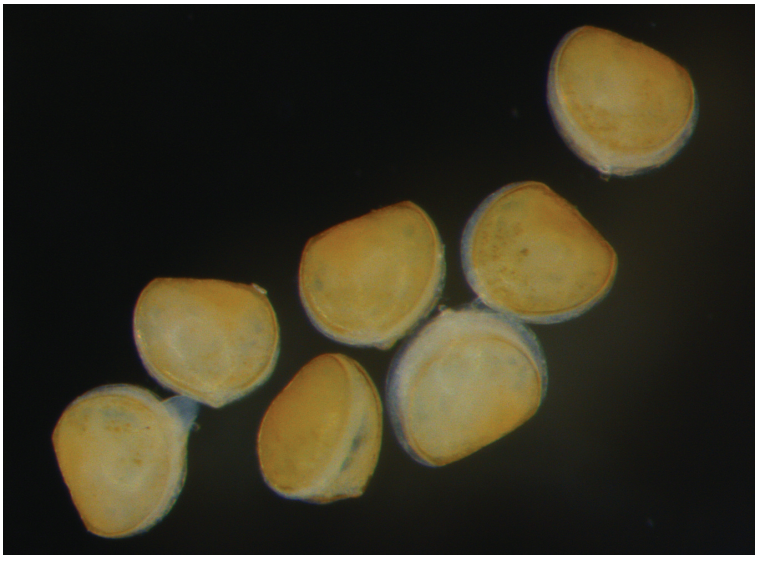

Figure 12. Juvenile *Velesunio* sp.

2. The chemical stock solution, along with 4 mL of 0.5 M HEPES stock solution (to achieve a 1 mM final concentration) and 4 mL of wet sediment (see below) are added to a 2 L volumetric flask and made up to 2 L with filtered MCW (testing in SSW has not been trialled for this species). The presence of sediment provides optimal conditions for feeding and growth (Hudson and Isom, 1984).

3. The solution is transferred to a 2 L HDPE bottle and the pH of each treatment is checked and adjusted drop wise with 0.05 M NaOH, or 0.05 M H_2SO_4 to ~pH 6.0.

TEST PREPARATION

Sediment preparation

1. ~0.28 g of dry sediment sieved to <63 µm is weighed out for each treatment (enough sediment to reach a turbidity of 100 NTU for each 2 L treatment).

2. The sediment is put into 5 mL plastic vials with lids and placed in an oven at 60°C for 24 to 48 h to reduce the presence of parasites.

3. 4 mL of MCW is added to each vial containing dry sediment and shaken by hand immediately prior to adding to the 2 L flask when mixing each test concentration.

Glochidia isolation

1. In the laboratory, glochidia are isolated from female mussels by placing each adult mussel into a separate clear, round polypropylene container (~10 cm diameter x 4 cm depth), and adding water from the collection site to each container, but leaving the top of each mussel shell slightly exposed (~2 cm depth).

2. The containers are covered and placed overnight in a constant temperature incubator (~27.5°C). The reduced water volume induces a stress response in gravid female mussels, causing them to expel glochidia from the inner gills, but without harm to the mussels.

Viability testing

1. Mature glochidia isolated from each mussel (preferably from a minimum of three adults) are assessed for viability by exposing a subsample (~50 to 100) of glochidia from each mussel to a concentrated salt solution (240 g/L NaCl), and counting the number of closed and open glochidia before and after exposure according to the following formula adapted from ASTM (2006):

$$\% \text{ survival} = 100 \times \frac{(\#\text{ closed after NaCl added} - \#\text{ closed before NaCl added})}{(\text{Total }\#\text{ open and closed after NaCL added})}$$

2. The sub-samples are discarded, and remaining glochidia pooled into a 400 mL crystallising dish and gradually acclimated to FDTW at a temperature of ~26 to 27°C for at least 3 h to prepare for the host fish exposure in filtered tap water.

3. Viable mature glochidia are used for host-fish exposure within 24 h of isolation from female adults, and only glochidia that have achieved >80% viability (preferably >90%) are used, as recommended in ASTM (2006).

Host fish exposure

1. The northern trout gudgeon, *Mogurnda mogurnda* (of ~15 cm length or approximately 6 months old), is used to host glochidia while they transform into juveniles. Host fish are

exposed to glochidia by placing two *M. mogurnda* and the glochidia into a 5 L plastic beaker containing 4 L of vigorously-aerated filtered tap water.

2. 10 x 25 µL subsamples of the glochidia sample are taken and counted to calculate the density of the sample. The volume of sample required to add 25,000 to 65,000 glochidia to the beaker, is calculated and the glochidia mixed into the beaker.
3. The fish are exposed for 35 minutes, and the water agitated by moving the air stone through the water column every 10 minutes (at 10, 20, and 30 minutes) to encourage the fish to move, and to ensure glochidia are suspended throughout the water column.
4. The fish are transferred gently using a wide mesh net (so that any remaining open glochidia are not transferred) into a 75 L plastic tub containing ~60 L of FDTW at ambient temperature (~27°C).
5. Fish are fed commercial fish pellets daily (Hikari® sinking carnivore pellets, Kyorin co. Ltd, Japan). Food can be minimised in the few days prior to release of juveniles so that there is less waste in contact with the excysted juvenile mussels.
6. One-third of the water is exchanged daily by removing 20 L from the tubs and replacing with new FDTW.
7. Fish waste falling to the bottom of tubs is siphoned through a 63 µm stainless steel sieve from d 5 onwards to collect post-excysted or 'released' juvenile mussels.
8. Fish are held in the tubs for up to 12 d, until the required juveniles had been collected, then returned to their original holding aquaria.

Obtaining juvenile mussels

1. Newly excysted juvenile mussels are collected by siphoning the content on the bottom of the fish tub through a 63 µm stainless steel sieve. Mussels are rinsed gently from the sieve with filtered tap water into a 400 mL crystallising dish.
2. Waste matter is removed from the water in the watch glass with a plastic pipette.
3. Juveniles are acclimated to the test diluent for ~4 h by replacing half the filtered tap water with the test diluent at the start of every hour.
4. A sample of 30 juvenile mussels are measured under the microscope to obtain an average starting length.
5. Juveniles are used in testing within 24 h of release from the fish.

TEST PROCEDURE

Day 0

1. 175 mL round, lidded polyethylene containers are set up on Perspex trays and labelled.
2. The turbidity of 23 test solutions is measured with the turbidity probe (TPS 90-FLT portable unit), to check that turbidity is ~100 NTU.
3. Algae (*Chlorella* sp., to achieve a final concentration of 8.0 x 10^4 cells/mL) is pipetted into each test container before dispensing 100 mL of test solution.

4. 50 mL of each test solution is dispensed into water parameter vials along with an adjusted volume of algae (half of that required for 100 mL).
5. The test containers and water parameter vials are transferred to the test incubator and left for at least 2 h to warm to test temperature.
6. The pH of new water in the water parameter vials is measured and adjusted to pH 6.0 if the difference in pH is >0.1, by adding 0.01 M H_2SO_4 or 0.05 M NaOH dropwise. The amount of drops needed to adjust the 50 mL water parameter vials is doubled to adjust the 100 mL test containers.
7. The test containers are removed from the incubator and the pH adjusted if necessary.
8. The trays of test containers are placed on a warming tray and juveniles are selected under a stereo-microscope (Leica MZ8), and transferred into test solutions using a 1-mm diameter Pasteur pipette. The criteria for selection of juveniles are an opaque appearance, the presence of internal organs observed through the part-translucent shell and pedal gape, and a moving foot.
9. Once each container has 10 juveniles, the lids are replaced and containers are arranged randomly on Perspex trays and returned to the incubator (27.5 ± 1°C).

Day 2, 4, 6, 8, 10, 12

1. Water changes are performed every second day.
2. A duplicate set of labelled test containers is set up on new trays.
3. The required volume of algae is added to each test container and water parameter vial, and the test solutions dispensed.
4. Waters are acclimated to the test temperature over ~2 h.
5. Water quality (EC, pH and DO) of each new water parameter vial is measured, and the pH adjusted to pH 6.0 if the difference in pH is >0.1. The pH of the test containers is then adjusted if necessary.
6. The trays of test containers are removed from the incubator and placed on warming trays.
7. For each test concentration, remove ~5 mL of overlying undisturbed test water from each replicate into a well of a 6-well plate using a plastic pipette. The plastic pipette is then used to gently remove juveniles and sediment stuck to the bottom of each test container.
8. The solution containing the juveniles is tipped into a watch glass.
9. Juveniles are pipetted from the watch glass into a 6-well plate, using a plastic pipette.
10. Following microscope observations, juveniles are pipetted gently into new water.
11. Pour ~40 mL of old water from each replicate into the water parameter vial.
12. Measure EC, pH and DO of the old water.
13. Rinse test containers and dry to be re-used for the following transfer.

Day 8 and 14: Growth measurements

Mussels need to be photographed with a microscope-camera set up, and length then analysed using

image analysis software. At *eriss*, mussels are photographed with a Leica M205C microscope and camera setup and length is measured simultaneously using the Distance Line Tool Analysis software module function (Leica application suite version 4.6.1).

On Day 8, (during the water change) juveniles are pipetted into a 6-well plate containing undisturbed water from each test container and photographed under the microscope before placing into new waters.

On Day 14, (end of test) juveniles are pipetted into a 6-well plate containing 70% ethanol. This enables quicker and more accurate measurements because the mussels do not move around. Following final measurements, mussels are discarded.

ANALYSIS OF TEST DATA

The mean starting length of the mussels is subtracted from the length of each individual mussel at the completion of the test. The growth rate of each individual mussel is calculated using the following formula:

$$\frac{\boldsymbol{Mussel\ length_{D14} - Mussel\ length_{D0}}}{\mathbf{14}}$$

A mean growth rate is calculated for each replicate and each treatment. The mean growth rate of each treatment is presented as a function of the control response and plotted against measured toxicant concentrations or effluent dilutions. The dataset is considered valid if >80% of the control mussels are alive and the growth rate of the control mussels is between 12.8 and 31.9 µm/d (average ~21 µm/d). Growth rate data are then analysed as described in the Generic Methodology section.

MOGURNDA MOGURNDA

Mogurnda mogurnda (commonly known as the purple-spotted gudgeon or the northern trout gudgeon) is a carnivorous fish species which is found throughout tropical northern Australia (Figure 13, Allen et al. 2002). Wild individuals used as broodstock have been obtained through multiple collections from a wide variety of habitats within the Alligator Rivers Region, including floodplain billabongs, lowland shallow backflow billabongs, lowland sandy creeks, channel billabongs and escarpment main-channel water bodies (Bishop et al. 2001). Broodstock were collected most recently in 2017, and maintained within the ecotoxicology laboratory of eriss. Permission to breed and use *M. mogurnda* for ecotoxicity testing was granted through the Charles Darwin University Animal Ethics committee (licence number A18020).

Mogurnda mogurnda have a maximum size of approximately 12 cm. They are coloured dark brown to purplish dorsally, lighter on their sides grading to white or cream on the belly and underside of the head. They have a series of vertically elongate, dark brown blotches or bars overlaid with numerous red and purple spots (sometimes arranged in horizontal rows) along the mid-side of the body. The head has three red-brown, diagonal bands radiating from the lower and rear edges of the eye across the cheek and gill cover. The fins are purple to yellow-orange, ornamented with red spots. In the breeding season, the purple and red spots become more intense, particularly in the male. Males may also develop intense blue colouration during courtship, while females may develop a yelloworange tinge to the abdomen.

ORGANISM CULTURE

Aquaria

Breeding pairs are kept in flow-through 288 L aquaria in a temperature controlled room (28°C) with a 12:12 h light:dark cycle. Overhead lighting (cool white) is suspended above the tanks to provide a light intensity of ~20-30 µmol photons/m^2/s. As *M. mogurnda* are a timid species, black mesh is used to cover the sides and the back half of the top of the aquaria, to provide the fish with access to both light and dark areas in the tank. Flow rate to the broodstock aquaria is set at 200 mL/min so that the entire tank volume is replaced within 24 h. The tanks are continuously supplied with FDTW that has passed through cartridge filters to remove solids and activated carbon filters to remove trace metals. The aquaria are given a thorough clean on at least a fortnightly basis by scrubbing the internal surfaces of the tank and removing debris with a gravel cleaning siphon tube. This cleaning and water replacement can stimulate courtship and subsequent spawning.

Figure 13.

Left: colouration of northern trout gudgeon (*Mogurnda mogurnda*).

Right: male fanning eggs.

Feeding

Gudgeons are fed once a day and appear to feed better in the afternoons. When gudgeon eggs are required for testing, fish are fed daily on a diet consisting of live earth worms and a commercial fish pellet (Hikari sinking carnivore pellets). This diet provides sufficient nutrition to the fish, and the provision of live food encourages regular breeding. If fish eggs are not required for testing, then fish are fed earthworms once a week and pellets at all other times. On weekends, it is generally more convenient to feed pellets to the fish. Juveniles are also occasionally fed leftover *Artemia*. The northern trout gudgeon is a timid fish, so the best approach to feeding is to move back from the tank after dropping the food in, to encourage fish to come forward and feed.

Breeding

Establishing compatible breeding pairs is largely a process of trial and error. It is recommended when trying to establish breeding pairs to allocate one female to two males. It is best to ensure they are from different genetic stock to avoid inbreeding and that the fish are similar in size to avoid bullying. If the female selects a breeding partner, the redundant male may be removed, particularly if the two males are aggressive towards each other.

Once breeding pairs have been established, daily observation during feeding is required to predict when a batch of eggs will be produced. This can be observed in both the behavioural and physical characteristics of the fish. These include courtship behaviour, in which the adults swim very closely together, accompanied by distinct golden colouration on the abdomen of the breeding female, and swelling and protrusion of both male and female papillae (Riethmuller et al. 2003).

The gudgeons will select, clean and guard a spawning site. Gudgeons seem to prefer to spawn on clean surfaces, so keeping small plastic plant pots (Figure 14) or smooth, flat plates (~13 x 13 cm) reserved for egg laying as clean as possible, will encourage spawning. Returning these items

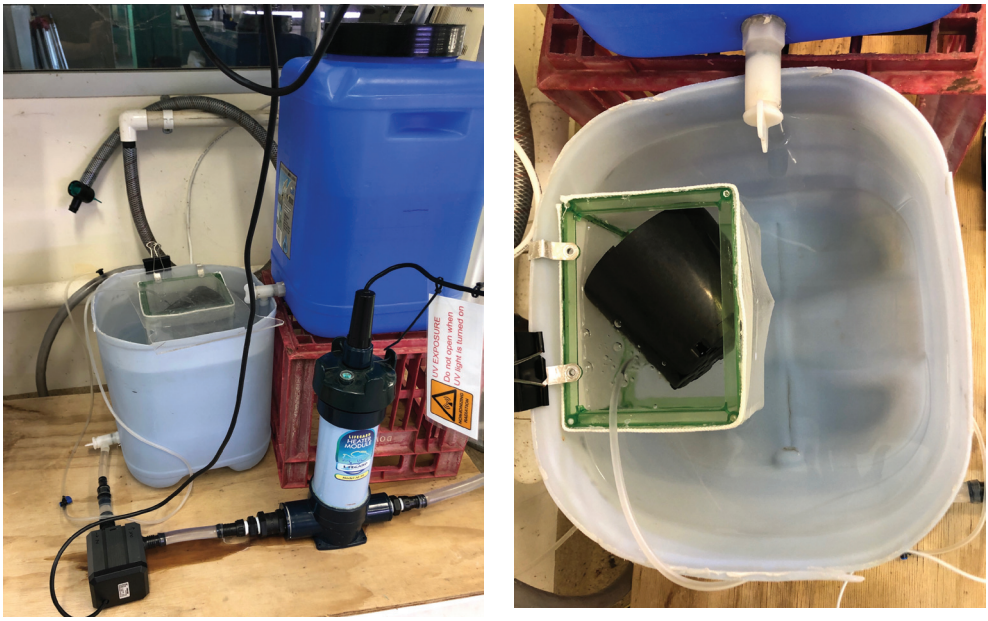

Figure 14. Left: Hatcher set up including UV steriliser. Right: Eggs on pot aerated in mesh enclosure.

after cleaning, to the same position and orientation within the tanks will minimise disruption to the gudgeons. The surfaces provided for egg laying need to be small enough to fit within the mesh enclosure of the hatching system (Figure 14). Gudgeons occasionally lay batches of eggs on the wall of the aquarium. These cannot be used in testing and are siphoned off and discarded.

The female lays a batch of eggs while the male fertilises them. The eggs are tubular in shape with transparent cases (Riethmuller et al. 2003). The egg batches range in size from 300 to 1000 eggs. After spawning, the female leaves the male to guard and care for the eggs, by fanning them with his pectoral fins to maintain a supply of oxygen and discourage fungal growth.

Juvenile gudgeons are raised in supplementary aquariums as broodstock replacements, should any individuals within a breeding pair/group die. When choosing broodstock it is imperative that the individual is healthy and free from abnormalities or deformities.

PRINCIPLE OF THE TEST

This chronic sub-lethal test method was developed from the chronic *M. mogurnda* toxicity test method developed by Cheng et al. (2010). Fry that are approximately 24 h-old (with a starting length of 4.28 ± 0.13 mm) are exposed to a range of toxicant concentrations for 7 d under controlled conditions. Growth (based on fry length) is measured for a sacrificial group of unexposed fry at the start of the test and then for all control and exposed fry at the completion of the test. The 7 d growth rate is compared to that of control (non-exposed) fry to generate an EC10 and EC50. The gudgeon is the only vertebrate species used at *eriss* representing the predatory trophic level in the biomonitoring suite.

TEST SOLUTIONS

Treatments are made up in 2 L volumes on the day of test start or the day prior, depending on whether equilibration of the test solutions is required. This provides enough test solution to last the 7 d test period.

TEST PREPARATION

Egg batches are removed from the parent aquarium within 24 h after laying and transferred (while submerged) to a separate hatching system (Figure 14). It is best to leave the eggs with the parents for as long as possible, provided the parents (or juvenile fish) do not eat the eggs. Ideally the hatching system should include a UV steriliser to prevent fungal growth and bacterial contamination of the eggs. Fry will usually hatch around 4 days after the eggs have been laid. It is recommended to monitor the eggs closely around the expected hatching time so that an accurate hatching time can be established.

Directly prior to the test start, 24 h-old fry are pipetted from the hatcher (using a large mouthed glass pipette) into a Petri dish of water from the hatcher at a density of ~50 fry per 50 mL Petri dish. Waiting for the fry to age 24 h before starting a test allows time for deformities to develop so that any affected fry are removed and not used in testing.

Healthy fry are selected from the Petri dish under the microscope and transferred to a new Petri dish which contains 50% hatcher water and 50% test diluent. This gradual acclimation reduces shock to the fry at the start of the test.

TEST PROCEDURE

Day 0

1. Three Petri dishes per treatment are set up on Perspex trays and labelled (A1, A2, A3, etc).
2. Starting with the control and working up the concentration gradient, 30 mL of test solution is dispensed into each Petri dish and the trays of dishes are left in an incubator for 1 h to warm to test temperature.
3. Fry can then be pipetted from the hatcher (as described above) while the test waters are warming.
4. Replicate Petri dishes of test solutions, and the Petri dishes containing the fry, are removed from the incubator and placed on warming trays.
5. Ten *M. mogurnda* fry are pipetted into each Petri dish with 30 mL of test solution. Either a wide-mouthed glass pipette can be used or plastic pipette with the tip cut and widened if necessary.
6. Two additional replicates (20 fry) are selected, euthanased (using MS222 – see below) and then measured. These fish are representative of the starting length of the fish. These data are then used to calculate the growth rate of the fish during the 7 d test.

Day 1 to 2

7. Test waters are dispensed to a second set of labelled Petri dishes, along with water parameter samples which are all warmed to test temperature in an incubator. Water parameters (EC, pH and DO) are measured prior to transferring fry to new water.
8. Fry are transferred to the Petri dishes of new test solutions under the microscope, with observations recorded on health and the presence of deformities. Any dead fry are removed. Trays of dishes are returned to the incubator as quickly as possible.
9. Following the transfer of fry, 24 h-old water is pooled for each treatment to measure EC, pH, DO and TAN.

Day 3 to 6

10. By day 3 the yolk sac of the fry is significantly reduced, and an alternate food source is needed. Previous work has demonstrated that this species showed no interest in feeding prior to day 3. Brine shrimp (*Artemia* sp.) are fed at a rate of 5 *Artemia*/mL of test solution, twice each day (see Appendix J). The first feed occurs 3 h prior to test start time. For this reason, a test start time of around midday makes it easier to incorporate the first feed at 9.00 am. Brine shrimp are directly pipetted into each Petri dish and replicates are returned to the incubator.
11. Test waters are dispensed and water quality parameters measured on the new water as described above.
12. At the time of test start, fry are observed and transferred to new test solutions. Fry are immediately fed again and are left to feed for 3 h, returning replicates to the incubator.
13. Water parameters are measured on the 24 h-old water.
14. After the fish have fed for 3 h, waste brine shrimp are removed using a Pasteur pipette. Replicates are returned to the incubator.

Day 7

15. On completion of the test, fry are euthanased prior to measuring length. An additional set of Petri dishes are set up and labelled in the same format as the test replicates. To each Petri dish, 30 mL of the test diluent and 1.5 mL of 10 g/L MS222 stock solution, (adjusted to pH 7 to pH 7.5), are added to achieve a 500 mg/L MS222 solution. Fish are transferred from the Petri dishes of test solutions to these Petri dishes containing MS222. Fish are placed in the euthanasia solution for 1 h prior to measuring length.

Sizing fry

To achieve a satisfactory level of accuracy, fry need to be photographed using a microscope-camera set up. Length can then be analysed using image analysis software. At *eriss*, fry are photographed using a Leica M205C microscope and camera, and measured using the Distance Line Tool in the Leica Application Suite V4.6 Software. Fry are removed from the MS222 solution and placed on a clean, dry Petri dish lid. All fry from the replicate are photographed in the same image with as much of the liquid removed as possible to reduce the magnifying effects of the water. Fish should be kept as straight as possible so that the length measurements are accurate. Length is defined as the direct distance between the tip of the mouth and the start of the caudal fin. The caudal fin is not included in the length measurement as it can be difficult to distinguish the end of the caudal fin in the saved images.

ANALYSIS OF TEST DATA

The growth rate of each individual fry is calculated using the following formula:

$$\frac{\boldsymbol{Fry\ length_{D7} - Fry\ length_{D0}}}{\mathbf{7}}$$

A mean growth rate is calculated for each replicate and each treatment. The mean growth rate of each treatment is presented as a function of the control response and plotted against measured toxicant concentrations or effluent dilutions. The entire dataset is considered acceptable if ≥80% of the control fry are alive at the completion of the test, with a minimum control growth rate of 0.085 mm/d (range observed in previous tests = 0.084-0.14 mm/d). Growth rate data are then analysed as described in the Generic Methodology section.

ACKNOWLEDGEMENTS

The authors acknowledge the contributions of previous and current researchers and technical staff at *eriss,* in particular, Alicia Hogan, Doug Holdway, Greg Rippon, Ross Hyne, Scott Markich, Claire Costello, Kim Cheng, Tida Nou, Nadine Riethmuller, Natasha Franklin, Caroline Camilleri, Catriona Turley, Constanza Buccella and Andrew Jansen. The authors also acknowledge the long-standing support and advice provided throughout the development of these methods from staff at CSIRO, in particular, Professor Jenny Stauber, Dr Merrin Adams and Dr Monique Binet Doyle.

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APPENDICES

APPENDIX A. COMPOSITION OF TEST DILUENT AND CULTURE WATER

Table A1. Measured inorganic composition of filtered (<0.45 µm) Synthetic Soft Water (SSW).

Chemical parameter	SSW[a]	Limit of Reporting
mg/L		
Ca	0.49 ± 0.02	0.1
K	0.38 ± 0.30	0.1
Mg	0.60 ± 0	0.1
Na	6.0 ± 0.8, 1.1 ± 0.1[b]	0.1
SO_4	3.5 ± 0.09	0.1
Cl	0.81 ± 0.04	0.1
NO_3 as N	0.82 ± 0.1[c], 0.25 ± 0.05[d]	0.005
PO_4 as P	0.015 ± 0.005[c], 0.03 ± 0.01[d]	0.005
µg/L		
Al	22 ± 3.0	0.1
Cd	0.03 ± 0.01	0.02
Cr	0.41 ± 0.03	0.1
Cu	0.65 ± 0.06	0.01
Fe	69 ± 3.4	20
Mn	9.5 ± 0.1	0.01
Ni	0.32 ± 0.04	0.01
Pb	0.25 ± 0.03	0.01
U	0.19 ± 0.07	0.001
Zn	4.0 ± 0.5	0.1

[a] Mean ± SE (n = 22) synthetic soft water (adjusted to pH 6 using H2SO4 or NaOH). Data taken from Trenfield et al. (2011). [b] Na in unadjusted SSW (n = 8, Pease et al. 2015). [c] Mean spiked nutrient concentrations (± SE) for *Chlorella* sp. tests; nominal N = 0.82 mg/L N and P = 0.012 mg/L, data from Trenfield et al. (submitted) and [d] *L. aequinoctialis* tests, nominal N = 0.23 mg/L N and P = 0.03 mg/L, data from Trenfield et al. (submitted).

Table A2. Stock solutions used to prepare synthetic soft water (SSW).

	Ingredient	Stock Solution (g/L)	Cation	Nominal conc of cation in stock (g /L)	Volume of stock per 20 L	Nominal concentration of cation in SSW
1	$NaHCO_3$	72.34	Na^+	19.79	1 mL	0.99 mg/L
2	$Al_2(SO_4)_3.18H_2O$*	17.26	Al^{3+}	1.398	1 mL	0.070 mg/L
3	$MgSO_4.7H_2O$	121.52	Mg^{2+}	16.186	1 mL	0.599 mg/L
4	$CaCl_2.2H_2O$	32.96	Ca^{2+}	8.985	1 mL	0.449 mg/L
5	KCl	14.09	K^+	7.389	1 mL	0.369 mg/L
6	$FeCl_3.6H_2O$	10	Fe^{3+}	2.066	1 mL	0.103 mg/L
7	Trace Element Solution	In 1 L add (g):			0.5 mL	
	$CuSO_4.5H_2O$	0.11	Cu^{2+}	0.028		1.095 µg/L
	$ZnSO_4.7H_2O$	0.123	Zn^{2+}	0.028		0.699 µg/L
	$Pb(NO_3)_2$	0.008	Pb^{2+}	0.005		0.125 µg/L
	$MnSO_4.H_2O$	1.188	Mn^{2+}	0.386		9.654 µg/L
	$UO_2SO_4.3H_2O$	0.007	UO_2^{2+}	0.0044		0.113 µg/L

* Requires heating to dissolve

Table A3. Composition of Filtered Darwin tap water (FDTW).

Chemical parameter	FDTW[a]	Limit of Reporting
mg/L		
Ca	6.1 ± 0.3	0.1
K	0.8 ± 0.1	0.1
Mg	3.8 ± 0.3	0.1
Na	2.9 ± 0.1	0.1
µg/L		
Al	8.6 ± 2.7	0.1
As	0.17 ± 0.01	0.05
B	12.5 ± 1.4	0.5
Ba	23 ± 1.2	0.02
Br	19 ± 3	1.0
Cd	<0.02	0.02
Co	0.03 ± 0.01	0.01
Cr	0.4 ± 0.04	0.1
Cu	4.2 ± 0.4	0.01
Fe	47 ± 9.2	20
Hg	<0.02	0.02
Mn	9.8 ± 2.0	0.01
Ni	0.2 ± 0.05	0.01
Pb	0.2 ± 0.03	0.01
Se	<0.2	0.2
U	0.02 ± 0.007	0.001
V	0.14 ± 0.01	0.05
Zn	3.5 ± 0.7	0.1

[a] Mean ± SE (n = 12). Unpublished chemistry data sampled by eriss over the year 2019.

APPENDIX B. PREPARATION OF MBL ALGAL CULTURE MEDIUM AND FLASK BUNGS

The algae are cultured in a modified MBL medium (Nichols 1973). It differs from the original medium in that $Na_2SiO_3.5H_2O$ is used in the trace metal stock solution in place of $Na_2MoO_4.2H_2O$. This replacement was made so that the medium would better resemble components of the natural waters from which the *Chlorella* sp. was originally collected (the waters of the region contain Si but not Mo). The stock solutions shown in Table B1 are added to Milli-Q® water in a 1 L volumetric flask. pH is adjusted to 7.2 ± 0.1 using 10% HCl. 100 mL of medium is dispensed to 10 x 250 mL flasks. The flasks are sealed with a bung that enables gas transfer (see Figure B1 and instructions below), and covered with aluminium foil. Flasks are autoclaved at 121°C and 120 kPa for 20 minutes and allowed to cool overnight before use.

PREPARATION OF MBL MEDIUM

Table B1. Stock solutions used to prepare MBL medium.

Ingredient		Stock Solution (g/L)	Volume of stock solution added to Milli-Q® water
1	Tris Base*	100	5 mL/L
2	$NaNO_3$	85.24	1 mL/L
3	$CaCl_2.2H_2O$	36.76	1 mL/L
4	$MgSO_4.7H_2O$	36.97	1 mL/L
5	$NaHCO_3$	12.6	1 mL/L
6	KH_2PO_4	8.72	1 mL/L
7	Na_2EDTA	4.36	1 mL/L
8	$FeCl_3.6H_2O$	0.727	1 mL/L
9	Vitamins Cyanocobalamin (Vitamin B12) Thiamine hydrochloride (Vitamin B1) d-Biotin (Vitamin H)	See below	1 mL/L
10	Trace metals	In 1 L add:	1 mL/L
	$CoCl_2.6H_2O$	10 mg	
	$CuSO_4.5H_2O$	9 mg	
	$Na_2SiO_3.5H_2O$	7 mg	
	$MnCl_2.4H_2O$	180 mg	
	$ZnSO_4.7H_2O$	22 mg	

* Tris base (Tris(hydroxymethyl)aminomethane)

Vitamin Stock

Biotin stock: Weigh 0.050 g biotin/500 mL Milli-Q® water (dissolve without heating).

Vitamin B12 stock: Weigh 0.025 g Vitamin B12/250 mL Milli-Q® water.

Note: These stocks can be stored frozen for many years.

Preparation of Vitamin stock:

Weigh 0.050 g thiamine into a dry 250 mL volumetric flask.

Add a few mL of Milli-Q® water to dissolve.

Add 2.5 mL of the above *Biotin stock* and 2.5 mL of the *Vitamin B12* stock.

Make up to 250 mL with Milli-Q®water. Use 1 mL of this stock per litre MBL medium.

Note: The Vitamin Stock is stored at 4°C and renewed every two years.

PREPARATION OF BUNGS

Bungs should be prepared from nonabsorbent cotton wool (e.g. Kapok) wrapped in a non-toxic fabric which can be autoclaved (e.g. Day-lee Towels) and secured with autoclave tape as follows. Care should be taken to ensure that the outer fabric and the filling do not contain traces of metals that may contaminate the algal media if there is contact between the medium and bungs.

1. Place a wad of cotton wool in the centre of the towel.
2. Using a double layer of towel, bring the towel up to enclose the cotton wool.
3. Wrap tape around the top to create a ‘handle’.
4. Cut off the excess towel.
5. Cover loosely with alfoil and place in the autoclave for sterilisation prior to use.

All bungs should fit firmly, but not tightly.

Figure B1. Left: Bungs for flasks. Right: Algal cultures on orbital shaker.

APPENDIX C. ALGAL CELL COUNTS USING A HAEMOCYTOMETER

COUNTING CELLS

If algal cells cannot be counted using an automated system such as a Coulter counter or flow cytometer, cell density can be determined microscopically using a haemocytometer.

The following description on loading and counting with the haemocytometer has been taken from Padovan (1992).

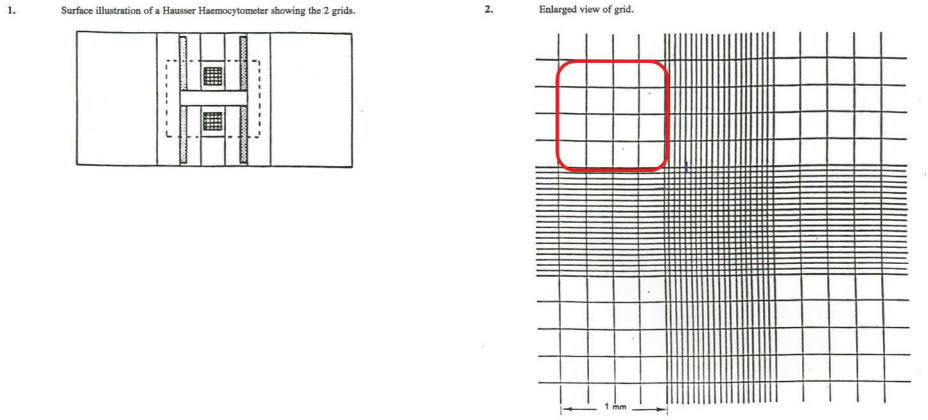
Surface and side diagrams of a haemocytometer are shown in Figure C1. Each slide is composed of two chambers, each containing a 3 x 3 mm ruled grid. Note that each grid is made up of nine squares of equal size, each 1 mm by 1 mm. The distance between the base of the grid and the bottom of the cover slip is 0.1 mm. Consequently, each 1 x 1 mm square grid has a volume of 0.1 mm^3.

Method

1. Rinse slide and cover slip with ethanol and wipe dry with lint-free towel.
2. Moisten the raised polished surfaces using a finger moistened with RO water and centre the cover slip on top. Press down firmly with thumbs over the raised areas to secure cover slip to slide (cover slip should remain attached even if slide is vigorously shaken!)
3. Place slide on a level surface and transfer a drop of well-mixed culture with a Pasteur pipette to the edge of the cover slip. The sample should be drawn into the filling chamber by capillary action. Remove pipette when the chamber is full to avoid over-filling (there should be no liquid in the depressions surrounding the three sides of the chamber or protruding from where the chamber was loaded). Re-mix the sample and load the other chamber.
4. Allow sufficient time for the cells to settle to the bottom of the chamber. Focus up and down to ensure there are no cells still in suspension. Both chambers should be counted as soon as possible as the samples will dry up and eventually start contracting. This should be avoided and the chamber rinsed and re-loaded if it occurs.
5. The choice of grid to use while counting is a function of cell density and the operator. At very low cell densities, the entire chamber can be counted. At higher densities, a smaller part of the chamber is counted, e.g. the squares making up the outer four corners of the grid (red square in Figure C1). At very high densities, count the centre square or its divisions. If the density is too high, the sample should be diluted prior to loading the cell.
6. The general rules to bear in mind in selecting which squares to count are: i) if possible, at least 100 cells should be counted per chamber, and ii) count cells from as wide an area as possible. For example if a 1 x 1 mm corner square (shown in Figure C1, composed of 16 smaller 0.05 x 0.05 mm squares) is sufficient to give a count of 100 cells, it would be better to count four of the 1 x 1 mm squares in each of the four corners.
7. Complications arise when cells lie on a line that borders the square being counted. Such cases need to be treated in a consistent manner by deciding which of the two vertical edges and which of the two horizontal edges are active (i.e. will have any cells touching them included in the count). The other two non-active sides will not have any touching cells counted. The choice of active sides is an arbitrary one, but one which should be

rigorously adhered to during any count. This rule applies whether the whole chamber or the smallest possible division is being counted.

Figure C1. Layout of counting cells on a haemocytometer.



Cell density determination

As mentioned earlier, each 3 x 3 mm grid can be divided into nine smaller 1 x 1 mm grids of equal size, where each smaller grid has a volume of 1 x 10^{-4} cm^3. Therefore the number of cells in any one of the nine smaller grids multiplied by 10^4 will give the number of cells per mL in the original solution (1 mL = 1 cm^3 = 1 mm^6 therefore 1 cm^3 divided by 0.1 mm^3 equals 1 x 10^4). All other calculations are variations of the same theme.

If appropriate, this value is then corrected for any dilution the culture may have undergone prior to loading the chambers.

Example

A 200 µL sample of an algal suspension is diluted to 50 mL for counting (1:250 dilution). The haemocytometer is loaded and four (1 x 1 mm) grids within each chamber are counted and summed. This is repeated three more times (i.e. two chambers on the haemocytometer are filled and counted twice) and the total count for each chamber is recorded as below.

a) 191

b) 173

c) 174

d) 180

The mean of these four counts is taken = 179.5

The mean is then divided by four (because four smaller grids within each chamber were counted) to give us the cell density of the suspension that was counted (x 10^4 cells/mL).

= 44.88 x 10^4 cells/mL

This cell density is then multiplied by 250 (i.e. 50 mL/0.2 mL) to account for the dilution of the original suspension.

= 1.12 x 10^8 cells/mL

APPENDIX D. CALCULATING ALGAL GROWTH

Table D1. Spreadsheet for calculating algal growth.

Test # **Date:** **Flow cytometer counts (cells/mL x E + 4)**

Treatment	Replicate	Day 0	Day 1	Day 2	Day 3	Slope	Growth Rate (doublings/day)	Mean	Pearson	% Control	Mean %
A	1	0.30		8.80	34.20	0.06644	2.30		100%	100%	
	2	0.30		8.40	33.30	0.06598	2.28	2.30	100%	99%	100%
	3	0.30		9.20	34.40	0.06664	2.31		100%	100%	
				Mean control growth rate =		***0.06635***					
B	1	0.30		9.80	36.10	0.06740	2.33		100%	102%	
	2	0.30		9.60	36.40	0.06744	2.34	2.34	100%	102%	102%
	3	0.30		9.40	37.90	0.06786	2.35		100%	102%	
				Mean growth rate =		***0.06757***					
C	1	0.30		7.20	32.40	0.06520	2.26		100%	98%	
	2	0.30		8.20	33.80	0.06609	2.29	2.22	100%	100%	99%
	3	0.30		5.00	25.00	0.06103	2.11		100%	92%	
				Mean growth rate =		***0.06410***					

Table D2. Log values used to calculate slope value in table above.

	Ln of Growth Rates						
	Day 0	Day 1	Day 2	Day 3	Below values for formula reference only		
A	8.006368		11.38509	12.74257	0	48	72
	8.006368		11.33857	12.7159	0	48	72
	8.006368		11.42954	12.7484	0	48	72
B	8.006368		11.49272	12.79663	0	48	72
	8.006368		11.4721	12.80491	0	48	72
	8.006368		11.45105	12.84529	0	48	72
C	8.006368		11.18442	12.6885	0	48	72
	8.006368		11.31447	12.7308	0	48	72
	8.006368		10.81978	12.42922	0	48	72

Table D3. Percent coefficient of variation (CV) for each treatment.

		Growth Rate (dblngs/day)			
		Mean	Std dev	%CV	SE
A	2.30 2.28 2.31	2.30	0.01	0.51	0.00674
B	2.33 2.34 2.35	2.34	0.00	0.04	0.00053
C	2.26 2.29 2.11	2.22	0.02	0.98	0.01259

APPENDIX E. CALCULATION OF ALGAL GROWTH CONSTANT AND DOUBLING TIME

(Modified from calculations prepared by Merrin Adams, Jenny Stauber, Barry Lumsden and Mark Florence, CSIRO Land and Water)

(Andersen 2005, Shuler et al. 2017)

SPECIFIC GROWTH RATE *N*

At any time *t* (in hours), the number of cells (*N*) is expressed as

$$N = N_0 e^{\mu t} \quad (1)$$

where N_0 is the initial number of cells, and μ is the specific rate constant for the cell division.

The actual rate is given by

$$\text{Rate} = \frac{dN}{dt} \quad (2)$$

i.e. the change in number of cells, *dN*, over a period of time, *dt*

Taking the natural log of equation 1 and further simplifying gives:

$$\mu = \frac{1}{t}\left(\ln \frac{N}{N_0}\right), \quad (3)$$

ESTIMATING THE SPECIFIC GROWTH RATE BY LINEAR REGRESSION

If , $\mu = \frac{1}{t}\left(\ln \frac{N}{N_0}\right)$. (Equation 3)

Rearranging Equation 3 shows that the specific growth rate, μ, is the slope of ln *N* vs. *t* (hours), the graph used for calculating the rate constant for *Chlorella* growth by linear regression.

$$\mu t = \ln N - \ln N_0$$

$$\ln N = \mu t + \ln N_0 \quad (4)$$

In the spreadsheet in Appendix D1, the slope is calculated using the SLOPE function in Excel.

Note that if the regression of $\log_{10} N$ vs *t* is used to calculate the specific growth rate, the slope must be multiplied by 2.303 to give the correct value for μ.

DOUBLINGS PER DAY AND RATE OF DOUBLING

Using Equation 3: $\mu = \frac{1}{t}\left(\ln \frac{N}{N_0}\right)$. which rearranges to : $t = \frac{1}{\mu}\left(\ln \frac{N}{N_0}\right)$,

The doubling time (hours) for the number of cells to double yield (i.e. grow from N_0 to $2N_0$) is t_{2x}.

$$t_{2x} = \frac{1}{\mu}\left(\ln \frac{2N_0}{N_0}\right)$$

Since $\log \frac{2\cancel{N_0}}{\cancel{N_0}} = \ln 2$, this simplifies to:

Doubling time = $t_{2x} = \frac{1}{\mu}(\ln 2)$ hours (5)

Now, Rate of doubling = $\frac{1}{t_{2x}}$, which is the reciprocal of Equation 5.

Rate of doubling = $\mu \frac{1}{\ln 2}$ h^{-1} (6)

Since ln 2 = 0.693, 1/ln 2 = 1.443

$$\textbf{Rate of doubling} = \frac{1}{t_{2x}} = \mu \times 1.443\ h^{-1}$$

$$= \mu \times 1.443 \times 24\ day^{-1} \quad (7)$$

APPENDIX F. CAAC CULTURE MEDIUM FOR *LEMNA*

1. Weigh out 2.05 g sucrose into a plastic weigh boat and add to a partially filled 1 L flask of Milli-Q® water. Shake the flask to dissolve the sucrose. (Do not keep sucrose as a stock solution as it is prone to bacterial contamination).
2. Add the appropriate amount of the stock solutions in Table F1 to the volumetric flask. Make flask up to 1 L volume. Stock solutions should be stored at 4°C, and will require replacing at 18 to 24 month intervals.
3. Adjust medium to pH 6.0 ± 0.15 using 0.178 M KOH (1 g KOH in 100 mL Milli-Q® water). Usually around 60 to 70 drops of KOH are required per 1 L of medium.
4. Pour CAAC medium into 10 x 250 mL flasks, such that there is 100 mL per flask.
5. Use a bung to plug the top of each flask. Cover the bung and neck of flask with alfoil. Record the date the medium is autoclaved and medium type on a strip of autoclave tape and place strip on the Alfoil cover.
6. Autoclave at 121°C for 20 min and allow to cool overnight before inoculating.

Table F1. Stock solutions used to prepare CAAC medium.

Ingredient		Stock Solution (g/L)	Volume of stock solution added to Milli-Q® water
1	KH_2PO_4	50.32	5 mL/L
2	KNO_3	88.9	5 mL/L
3	$Ca(NO_3)_2.4H_2O$	94.4	5 mL/L
4	$MgSO_4.7H_2O$	50	5 mL/L
5	EDTA	9	500 mL/L
6	Tartaric acid	3	500 mL/L
	Micronutrients	**In 1 L add:**	
	H_3BO_3	2.86	
	$ZnSO_4.7H_2O$	0.22	
7	$Na_2MoO_4.2H_2O$	0.12	500 mL/L
	$CuSO_4.5H_2O$	0.08	
	$MnCl_2.4H_2O$	3.62	
	$FeCl_3.6H_2O$	5.4	

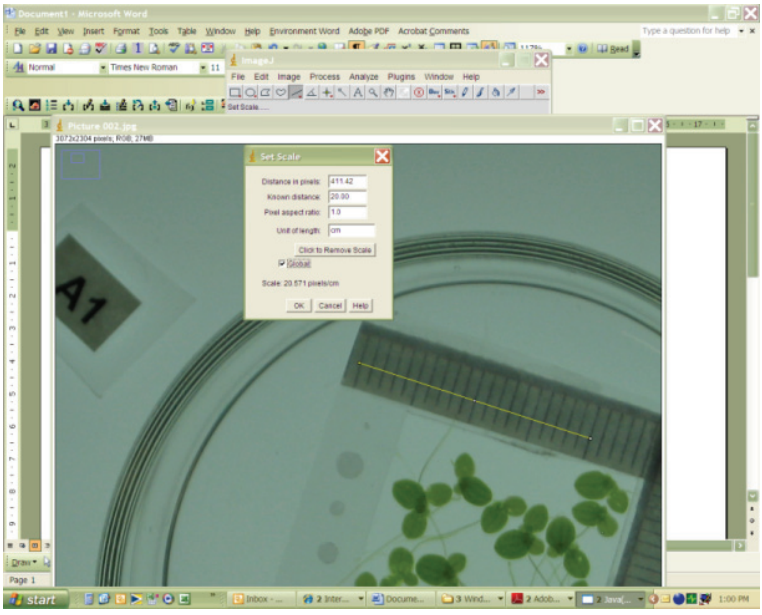
APPENDIX G. MEASURING FROND SURFACE AREA IN IMAGEJ

The ImageJ software can be downloaded from http://rsbweb.nih.gov/ij/index.html.

1. Open the picture in ImageJ (v. 1.51k was used for this example).
    a) File ® Open ® Select photo. (Note: It is easy to preview files and open them using the Windows photo browser and the "open with" function in the right click menu)

1. Set the scale using the line tool (Figure G1)
    a) Select the line tool (i.e. 5th box from the left under the menu bar). Draw a line on the scale of the floating frame (zoom in (i.e. ctrl and +) for more accuracy).
    b) Analyze ® Set scale. Add the length of your line in the Known distance box, and enter the units in the Unit of length box (e.g. cm).
    c) Check the global box so that all subsequent images will use this scale.
    d) Click OK.

Figure G1. Setting the scale



2. Choose Surface area (SA) as a measurement
    a) Analyze ® Set measurements. Select parameter(s) required (i.e. area). Check "Limit to threshold" box, and also the "Display label" box.

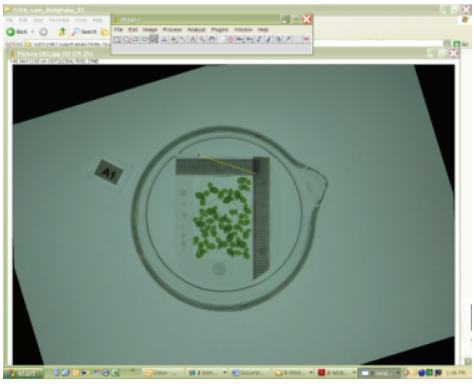


Figure G2. Rotated image

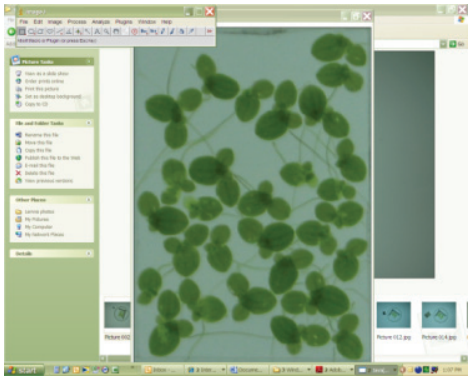


Figure G3. Cropped image

3. Rotate and Crop

    a) If needed, rotate the image (Image ® Transform ® Rotate; Figure G2) so that the plastic frame is squared and aligned.

    b) Select the required area using the Rectangle tool, and crop the photo (Image ® Crop; Figure G3). It is important to remove the scale and as much background as possible leaving only *Lemna* fronds.

4. Open the Threshold Colour tool.

    a) Open the dialogue box (Image ® Adjust ® Colour Threshold; Figure G4).

The Colour Threshold tool methods that could be used: i) HSB (Hue, Saturation and Brightness), ii) RGB (Red, Green, Blue), and iii) YUV (colour encoding system used for analogue television).

HSB system discriminates the *Lemna* leaves from the roots and was most useful for this method. Three histograms are shown and represent the HSB parameters, Hue (pure colour), Saturation (intensity of colour) and Brightness (relative to true colour). Make sure that the threshold colour is also set to red.

5. Set threshold for green pixels

    a) *Lemna* fronds have a green hue (hue = "pure" colour) – Set the bottom-sliding bar on the Brightness histogram to 255 so that the whole image goes red. Then set the bottom sliding bar on the Hue histogram to 120 (Figure G5). By moving the sliding bar to 120, only the *Lemna* should be red and the roots and background within the image are excluded.

6. Click 'Select' and a yellow border will appear around the fronds (Figure G6).

7. Collect the measurement data. Analyze → Measure (Ctrl+M). A results table will appear with the area in the units specified in step 2 (e.g. cm^2, Figure G7). The mean, min and max values of the pixels in each image are also automatically generated. This can be saved or cut and pasted into an Excel spreadsheet for analysis. To ensure that the column headers copy when you copy the data out of ImageJ and paste in to Excel, select the results box, click on Results → Options and under Results Table Options make sure that the 'Copy column headers' box is checked.

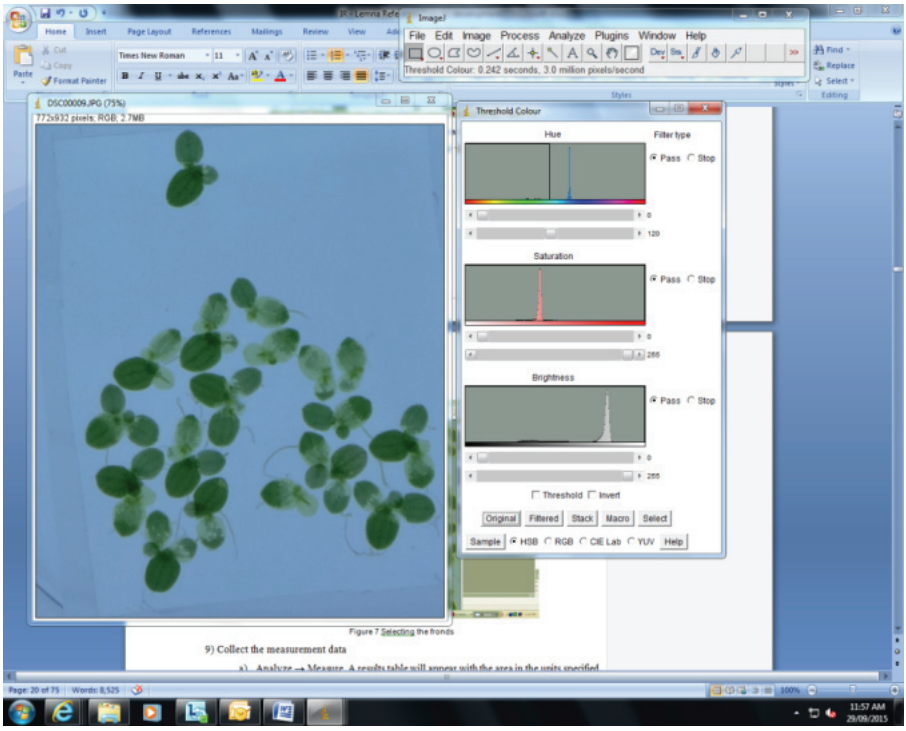


Figure G4. Threshold Colour

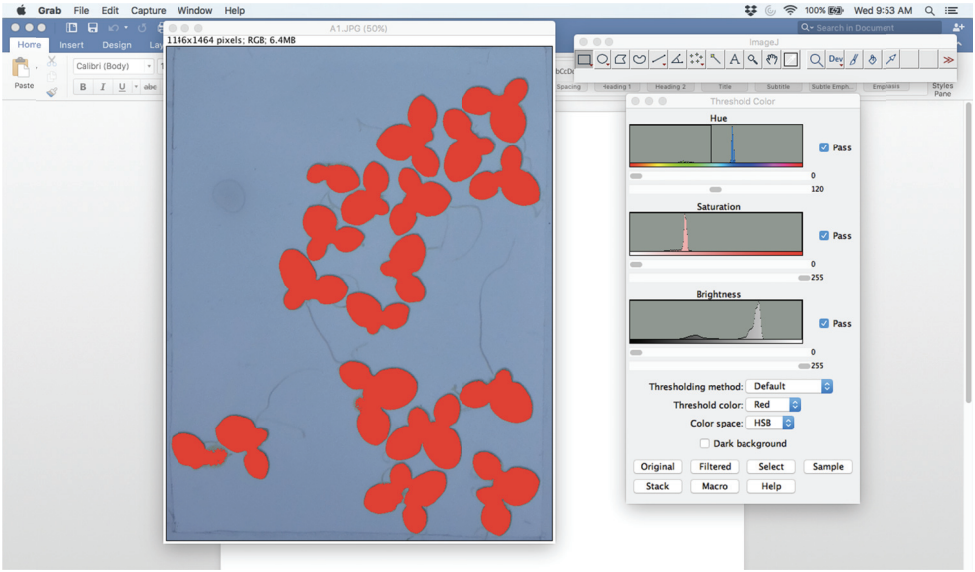


Figure G5. Setting HSB threshold

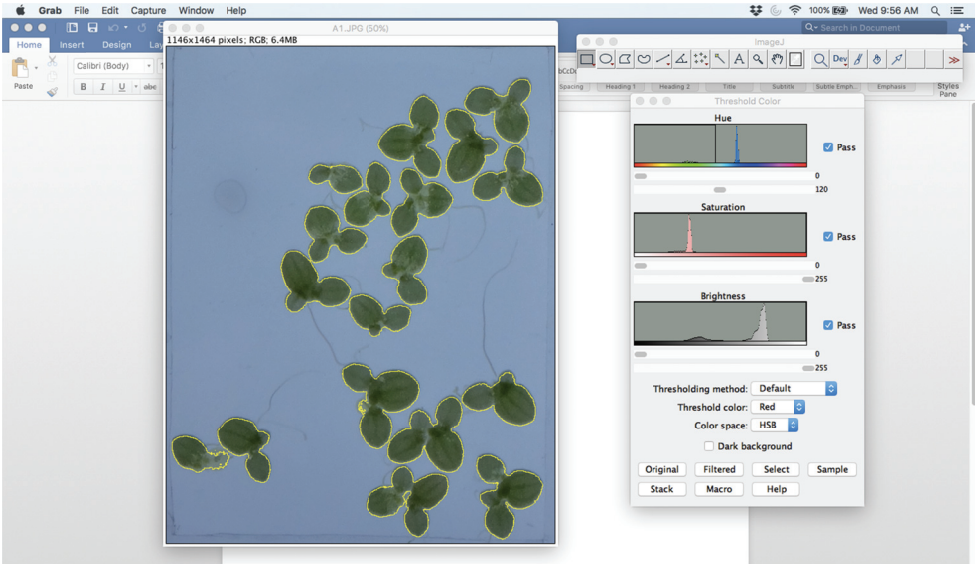


Figure G6. Selecting the fronds

Results

File Edit Font Results

	Label	Area	Mean	Min	Max
1	Lemna control 01.jpg	0.695	52.545	17	112
2	Lemna control 01.jpg	0.590	56.329	30	115
3	Lemna control 01.jpg	0.075	64.107	40	111
4	Lemna control 01.jpg	0.450	57.267	25	111
5	Lemna control 01.jpg	0.966	56.193	23	107
6	Lemna control 01.jpg	0.465	53.822	27	115
7	Lemna control 01.jpg	0.796	52.474	22	133
8	Lemna control 01.jpg	0.727	58.405	28	110
9	Lemna control 01.jpg	0.407	54.929	30	123
10	Lemna control 01.jpg	0.867	54.056	24	115
11	Lemna control 01.jpg	0.784	51.195	21	120

Figure G7. Example of a results table created in ImageJ.

APPENDIX H. PREPARING FERMENTED FOOD WITH VITAMINS

The cladocerans are fed a bacterial food source that is supplemented with vitamins, i.e. Fermented Food with Vitamins (FFV).

Safety

Microbiological testing of FFV has found it occasionally consists of *Bacillus cereus*, a gram-positive rod-shaped microbe which, under certain conditions, produces a toxin that can cause gastrointestinal illness.

The toxin itself has not been identified in FFV but as a precaution, FFV should be handled as an infectious agent to protect the health of laboratory staff. Specifically, vessels containing concentrated FFV need to be labelled appropriately, contaminated pipette tips need to be disposed of in yellow biohazard bins, appropriate PPE needs to be worn, i.e. laboratory coats and gloves, and strict laboratory hygiene should be observed.

Ingredients

- 5 g floating/sinking cichlid pellets (both Ocean Star International (OSI) brand pellets and API Cichlid pellets have been used).
- 0.25 g dried powdered alfalfa (weighed out from Blooms Super Alfalfa 1000 capsules; any excess alfalfa from a capsule is discarded).
- 250 mL Milli-Q® water.
- 0.3 g calcium pantothenate.
- 1.5 mL vitamin B_{12} stock solution (100 µg/L). Make this vitamin stock in a 100 mL volume, and dispense smaller amounts as required.

Method

1. Homogenise the cichlid pellets, alfalfa and water for 5 minutes in a blender.
2. Bubbleaerate this mixture at 24 to 28°C for no longer than three days, in a 1 L sediment (Imhoff) cone covered with a fine mesh to keep out dust, flies and to allow gases to be released.
3. Rinse the blender thoroughly with Elix® (RO) water followed by a rinse in high-purity Milli-Q® water.
4. Check the volume in the cone each day and top up to 250 mL as required, with Milli-Q® water. When fermenting, smell-test the mixture frequently to determine if harvesting is due.
5. When ready to harvest, the mixture will have a strong pungent odour. If it smells putrid, it has been fermenting too long and must be discarded.
6. Harvest mixture in the afternoon by draining through the bottom of the cone into a glass beaker. Cover with Parafilm® and place in a refrigerator at 4°C overnight.
7. Decant the clear, orange-brown supernatant into another beaker there should be approximately 150 mL. Discard any cloudy sludge that has settled at the base of the beaker.

8. Add the vitamin B_{12} and calcium pantothenate and mix thoroughly on a stir plate.
9. At this stage, carry out a visual check on the mixture by comparing its colour and density with a portion of the previous harvest. If they are similar, use the new batch immediately, or store frozen until needed. If the batches are not similar, the new batch must be tested for suitability by performing a cladoceran reproduction test in which >80% of adults must survive. The minimum total brood number must be >30, with the three broods being produced within 5 to 6 days of birth. If these standards cannot be met the new batch must be discarded (all test records must be kept).
10. Dispense 4.0 mL of FFV into 5 mL sterile plastic vials, cap, and label with date of preparation and freeze. Ensure that you use a pipette tip which contains a filter to avoid contamination of the pipette.
11. Record the date of preparation on the laboratory maintenance chart on the main laboratory bench.
12. Thoroughly wash the cone and any other apparatus that has come in contact with the FFV with Decon Neutracon detergent, rinse with RO water and then through a full dishwasher cycle at 60°C.
13. TOC analysis should be conducted on each batch to determine whether it is consistent with the TOC of previous batches. A TOC sample can be prepared by pipetting 30 µL FFV into 30 mL Milli-Q® water.

To use:

1. Remove one vial from the freezer and allow to thaw.
2. When dispensing, shake well to ensure thorough mixing, and add 30 µL to 30 mL of creek water.
3. A thawed vial must be refrigerated and discarded if not used within one week.

APPENDIX I. CULTURING ALGAE FOR CLADOCERAN FOOD

Cultures of algal food (using the green alga *Chlorella* sp.) are prepared as follows:

1. Autoclave approximately 1 L of MBL media in each of 4 to 5 x 2 L Erlenmeyer flasks with cotton wool bungs.
2. Allow flasks to cool overnight before inoculating
3. Using aseptic techniques, and ensuring that the starter culture is well mixed, inoculate each flask with **10 mL** of a week-old starter culture of *Chlorella* sp. in order to achieve a starting cell density of approximately 10^4 cells/mL.
4. Place the cultures on an orbital shaker (120 rpm) in a growth cabinet with a 12:12 h light:dark cycle at 29°C.
5. The culture should take approximately 10 to 12 days to reach a cell density of 2 x 10^6 cells/mL, at which time it can be harvested. A cell count can be performed to check this, but with experience, the correct harvest time can be determined by assessing the colour of the culture.

Algal cultures are harvested as follows:

Approximately 1 L of autoclaved diluent water is required to rinse and store the harvested algal cells. This should be prepared at least the day before the harvest so that it has cooled to room temperature before use.

1. Ensure your hands are contaminant-free by washing with soap and water, rinsing with Elix® or Milli-Q® water and wiping with Isowipes®.
2. The harvest is divided among four 600 mL centrifuge bottles.
3. Balance the bottles so that they are of equal mass and centrifuge them for 10 min at 2500 *g* and 4°C. Ensure that the centrifuge deceleration is set at as slow as possible (or the brake is off) otherwise the algae will be stirred up if the centrifuge stops rapidly.
4. Gently decant and discard the supernatant, leaving the concentrated algae at the base of the bottle (Figure I1).

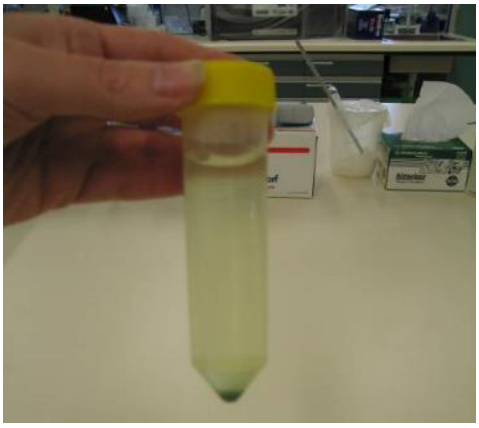


Figure I1. Centrifuge tube with pellet of Chlorella at base.

5. Repeat the previous three steps for any remaining harvest culture (which can be added to the concentrated algae in the four centrifuge bottles).
6. Divide the concentrated algae suspension between four 50 mL centrifuge tubes and top up with autoclaved diluent water.
7. Balance the tubes so that they are of equal mass and centrifuge them at 4°C for 7 min at 2500 RPM (which as a reference, is equivalent to 1050 *g*). The brake deceleration can be increased for this cycle as the conical base of the tubes prevents the algal pellet from being stirred up when the centrifuge stops rapidly and the brake saves a lot of time (Figure I1).
8. Decant the supernatant and half fill the tubes with autoclaved diluent water.
9. Vortex the tubes to mix the pellet with the sterile creek water, thereby rinsing the culture media from the cells (this step is important as the nutrient rich medium will make the stored cells more prone to fungal and bacterial contamination).
10. Top up and balance the tubes with the autoclaved diluent water and repeat the previous five steps three times to ensure that the algal cells are thoroughly rinsed. Wash and wipe your hands with isowipes each time you return to the centrifuge to handle the algae harvest.
11. Combine the algal pellets from all four tubes into a sterile 250 mL beaker, make up to approximately 100 mL with Milli-Q® and cover with Parafilm.
12. Determine the density of this suspension. If an automated cell count cannot be performed, the manual microscope method outlined in Appendix C can be used.
13. Dispense 4 mL aliquots of suspension into 5 mL sterile tubes (Figure I2). Although the algal culture is not sterile, dispense the aliquots in a laminar-flow cabinet using equipment that has been exposed to UV light for at least 30 min to maintain the highest possible level of cleanliness. Also, avoid touching the lids and tops of tubes and use a pipette tip with a filter to avoid splashback and potential contamination.
14. Store the dispensed algae in a refrigerator (4°C), and mark each tube with the date of harvest plus the volume (in µL) required to be added to a 30 mL flea culture to result in 6 x 106 algal cells/mL (Figure 4). This volume equals one unit (as described below).
15. Total organic carbon analyses should be conducted on each harvest to determine the consistency of the algal culture with previous batches.

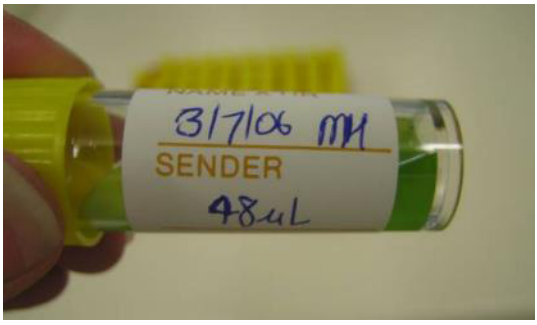


Figure I2. Vial containing 4 mL of *Chlorella* and labelled with date, initials and inoculation volume.

Below is a guide to calculating the volume of algae required for a test, and to decide when the next algae harvest cultures should be inoculated. Currently a new bulk culture is inoculated every two to three weeks to ensure a constant supply of fresh algae for the cladoceran cultures.

Assume that one unit of algae = 6 x 10^6 cells. The number of units per vial depends on the cell density and generally ranges between 50 to 200 units. This can be calculated for each batch by dividing 4 mL by the volume of one unit of algae (i.e. the volume that is dispensed into each flea vial).

The following is a guideline to the amount of algae required (Table I1).

Table I1. Units or vials of algae required for culturing or testing with cladocerans.

	Quantity
General culture	252 units (or 1–2 vials) per week
5 to 6-day reproduction tests (6 treatments or 60 flea vials)	360 units (or 2–3 vials) per test

APPENDIX J. BRINE SHRIMP CULTURE AND HARVEST

Brine shrimp (*Artemia* spp.) are used as food for many aquatic organisms, including *Hydra* and larval fish. Brine shrimp are cultured in the Ecotoxicology Laboratory to provide a continual supply of nauplii for the *Hydra*. The cultures are maintained in 1-L graduated hatching cones (Figure J1).

Method

1. A salt solution is made by dissolving approximately one and a half tablespoons of uniodised coarse pool salt or sea salt in ~1 L of FDTW (25 to 35 ppt salinity).
2. The cone is attached to an air-line and the tap on the stopper is opened to gently aerate the solution with oil-free compressed air. After the salt is fully dissolved, half a teaspoon (~5 g) of commercially-harvested, dried brine shrimp cysts are added.
3. The solution is continuously aerated to prevent the cysts from settling. The cysts hatch after 24 to 30 h at an incubation temperature of 27°C, with a 12:12 h light:dark cycle. Cysts should be stored long-term in the freezer and storage in the refrigerator should be minimised to a month.

Harvesting

4. The tap of the cone is turned off and the suspension is left for about five minutes, allowing the nauplii to collect at the base and the empty egg shells to float to the surface. *Artemia* are known to be phototactic (i.e. attracted to light), so if the nauplii are not concentrating at the bottom, cover the top section of the cone with dark plastic (or similar) to encourage the nauplii to swim towards the light at the lower end of the cone.
5. The airline is removed from the tap taking care not to dislodge the stopper tap in the process. The lower half of the cone volume is drained through a 250 µm mesh nylon net, ensuring that the top layer containing empty egg shells is not collected.

Figure J1. Brine shrimp (Imhoff) cones.

6. The nauplii captured in the net are rinsed thoroughly using FDTW, with a final rinse in the water that is being used for culturing or testing. They are re-suspended in ~100 mL of culture (or diluent) water in a beaker. If any unhatched cysts have been collected they will settle at the base of the beaker, while live nauplii will concentrate in a layer above the unhatched cysts.
7. Live nauplii are collected for feeding and are gently and evenly distributed across the *Hydra* culture dish with a Pasteur pipette.

Calculation of brine shrimp density

1. 24 h-old *Artemia* are fed to the larval fish at a rate of 5 *Artemia*/mL
2. *Artemia* are rinsed in a 250 µm nylon net and transferred to the diluent required for the test. Remove as many unhatched cysts from the solution as possible.
3. Stir the solution and remove five 25 µL sub-samples. Spread them across a clean dry Petri dish.
4. Count the number of *Artemia* in each 25 µL subsample.
5. Find the average and then multiply it by 40 to get *Artemia*/mL.
6. Use the following equation to calculate the volume of solution that needs to be added to each Petri dish.

$$volume = \frac{test\ solution\ volume\ \times Artemia/mL}{Artemia/mL\ in\ solution}$$

(where test solution volume = 30 mL and the required *Artemia*/mL = 5)

APPENDIX K. DIGITAL COUNTING OF SNAIL EGGS

EGG LAYING VIAL AND PLASTIC INSERT

During *in situ* snail testing, snails are contained in transparent vials. The components that make up the vials for testing are shown in Figure K1.

- **The vial:** Clear polycarbonate tubing 50.8 mm external diameter, 47.6 mm internal diameter, approximately 70 mm long.
- **Transparent plastic inserts:** Approximately 14.6 x 5.6 cm polycarbonate rectangles, pre-cut, to insert into each vial as a substrate for snails to lay eggs on. The inserts are removed and photographed when the test is terminated. Plastic was 0.8 mm thick and sourced from Bunnings, https://www.bunnings.com.au/suntuf-1-x-1m-x-0-8mm-clear-uv2-polycarbonate-sheet-handi-roll_p1010853.
- **Mesh pieces:** Approx. 8.5 x 8.5 cm squares of soft ~1 mm nylon mesh, Sourced from Rowe Scientific.
- **Clips:** 50 mm (55 mm external diameter) PVCu (unplasticised polyvinyl chloride) pipe, cut into 5 to 7 mm rings, 1/5th of ring circumference removed. Edges should be filed smooth to avoid tearing the mesh pieces.

Figure K1. Vial parts: vial, mesh, clips, plastic insert and tool to remove plastic inserts.

Before a test

The vials have a transparent plastic insert, and a mesh and clip fitted at one end ready for snail introduction on the starting date. Treatment and replicate numbers are marked in permanent marker on the outside of the plastic inserts so the number is facing inwards to the centre of the tube. The plastic insert is curled and positioned inside the tube, once it is within the tube, the remainder is pressed in. The mesh is held in place with a clip on one end of the tube and positioned flush against the insert. The clip and the clip edge are made flush with the end of the vial. The open end is sealed with a clip and mesh, making necessary adjustments to ensure the insert is flush against both clips (Figure K2).

Figure K2. Steps for placing the plastic insert into the test vial.

After each test

The plastic inserts are removed using PVC pipe with cloth cable-tied over the end to push the inserts out (Figure K3). This is achieved by placing a clip with no mesh inside the tube flush up against the insert, and pushing the clip with the cloth end of the PVC pipe. The insert is pushed until half is exposed, the remainder is removed by hand. Care is taken when pulling the insert completely out as the plastic in its rolled up position will spring open, potentially damaging the egg masses laid. To avoid this, the insert is kept coiled as it is pulled out, then slowly allowed to un-coil. Inserts with egg masses are placed in RO water whilst preparation for photography. Any dirt or foreign material on the inserts are removed using RO and squeeze bottle.

Figure K3. Steps to collect plastic inserts using removal tool

Setup for photography of snail egg masses

Items needed to photograph snail egg masses on plastic inserts include:

- Glass tank filled with RO water
- Three vice grips
- Light box (cool white fluorescent tube)
- SLR camera with 100-mm macro lens
- Camera tripod
- Clear plastic guard made from the same polycarbonate sheeting as the insert (28 x 11 cm)
- Black laminated card (to act as a border for the plastic insert)

The following set up would occur ideally in a dark room. The distance between the camera lens and the glass box will depend on what distance the camera needs to properly focus on the eggs to capture all egg masses clearly. In a semi-dark room, 76 cm was suitable (Figure K4). The light box should be positioned behind the glass box with a gap. This illuminates the cell walls of the egg masses, making them easier to define in imagery. The glass tank is filled with RO or Milli-Q® water rather than tap water to eliminate any interference of tap water with the egg masses and to prevent the build-up of chlorine, calcium, etc, on the tank, which leave white spots on the glass. A border for the plastic insert, which can be made out of black, laminated card is positioned at the front, on the outside of the tank. This reduces interference from other light sources or shadows in the room and improves contrast between the egg masses and the background.

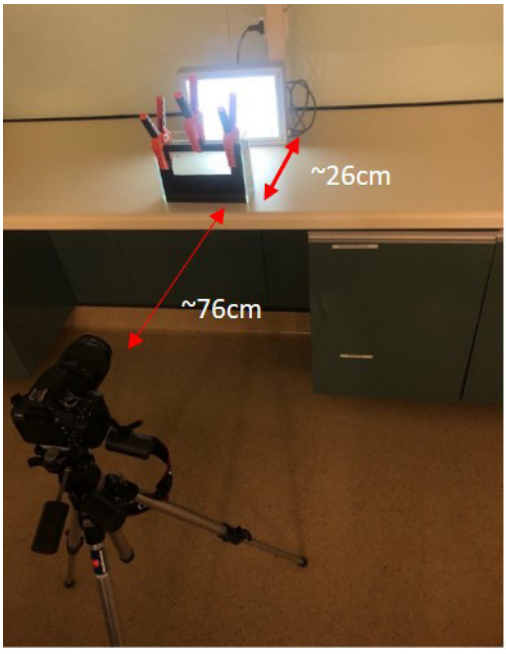


Figure K4. Camera setup for photographing snail egg masses on plastic inserts

Using a vice grip the plastic guard is positioned on the inside of the front of the glass tank and clamped on one side, ensuring the clamp is placed on the black border (Figure K5) and not on the clear section of glass. The plastic insert with egg masses is then inserted between the plastic guard and the glass so that the egg masses are facing the front of the tank. The water in the tank prevents the egg masses from getting stuck to the glass, or scratched by it. Once in position, the remaining two clamps are placed as seen below (Figure K5). The clamps are positioned over the black card and plastic guard, not on the insert with egg masses. This reduces the chance of damaging the egg masses and removes the clamps from images. The purpose of the guard is to ensure the insert is pushed evenly up against the glass so that the camera is focussed on all egg masses.

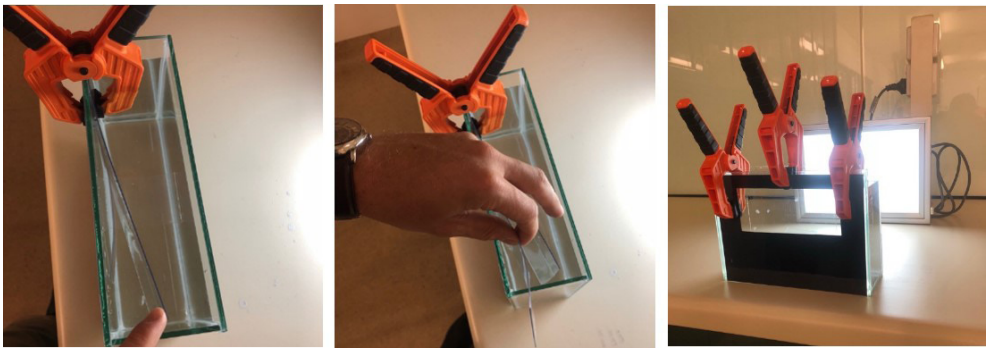


Figure K5. Left: Holding the plastic guard, centre: positioning the insert with egg masses, right: correct position for the vice grips

The camera, on the automatic setting [A^+], is focused on the egg masses by half-pressing the capture button. The camera will begin to zoom in and out whilst focusing the field of view. When it has focused, the camera will stop moving and beep, indicating an image is ready to be taken. The capture button is completely pressed down to take the image. For the first five images/replicates, the quality of images is checked by pressing the image preview button ▶ and zooming in on the egg masses using the buttons with magnifying glasses below them. The image may not appear to have clear resolution when it appears as it does in Figure K. When zooming in, the resolution becomes clear and the egg masses, eggs and embryos become clearly distinguishable (Figure K7). If they do not, the clamps are checked for equal distribution across the plastic guard to ensure the plastic

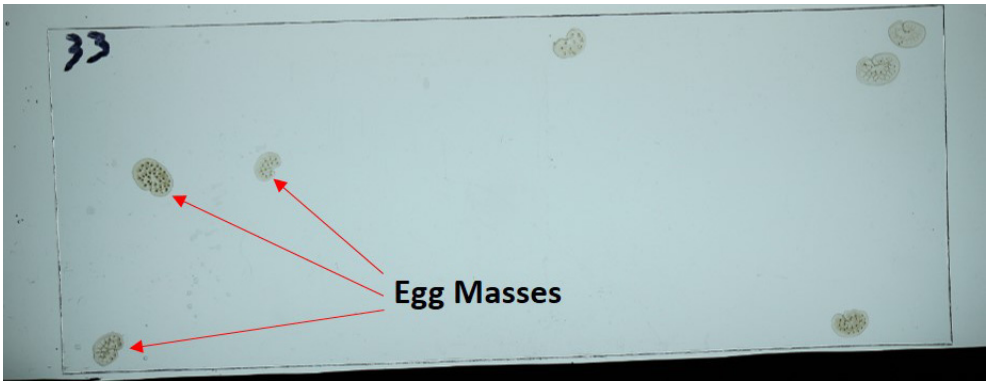


Figure K6. Egg masses on plastic insert.

insert with embryos is flat against the glass. The front of the tank should also be cleaned of smudges or droplets, as the camera will focus on these first rather than the eggs. The tripod also should be positioned with all legs equally distributing the weight of the camera (if the camera moves the image will blur). When five successive images are clear, there is no need to check the images or look down the camera lens for each replicate.

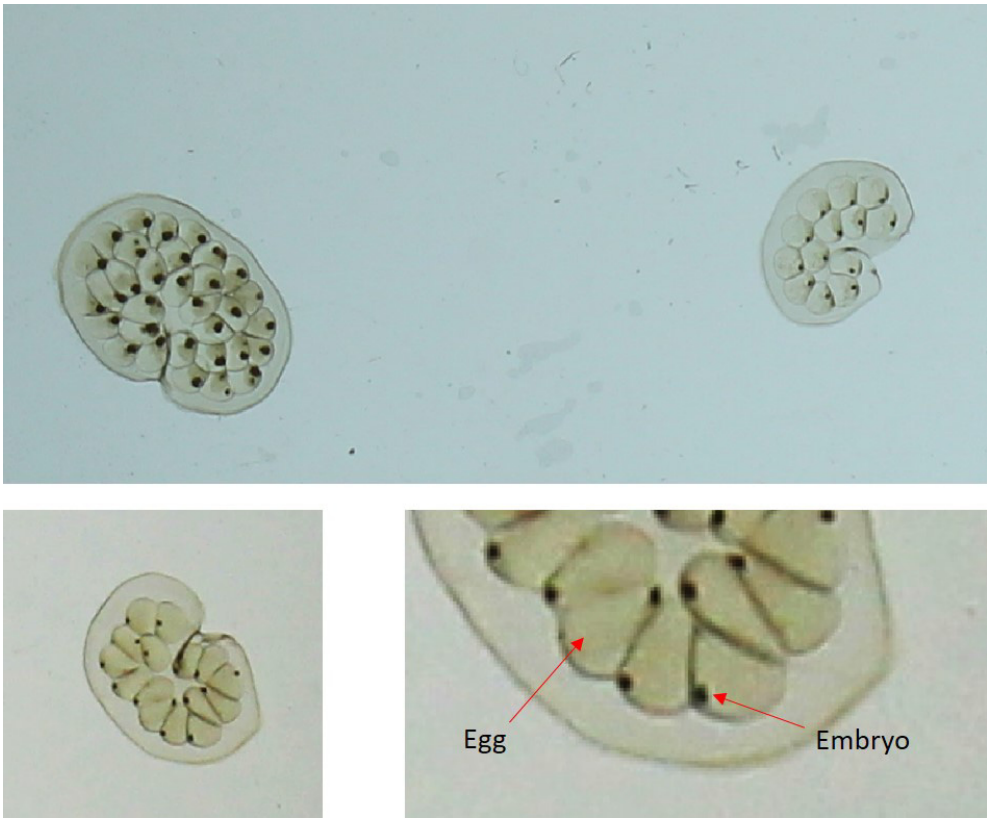


Figure K7. Egg masses (zoomed in).

DIGITAL IMAGE ANALYSIS

Eggs and egg masses are counted from photographs using the computer software package FIJI (v 1.52T), a form of Image J, cell counter tool.

Egg Counting

1. Open an image for counting using Fiji: File → Open → 'locate image in directory'.
2. Open the cell counter tool: Plugins → Analyse → Cell Counter.
3. Count the number of egg masses in the image then add that number of counter types using the 'add' button (or alternatively remove counter types from the list to result in the correct number of counter types).
4. Click Initialize then click on counter Type 1, then zoom in on the egg mass by holding the Ctrl button and using the scrolling wheel on the mouse (represented by the + symbol; if this is not the case, make sure you are not in the ImageJ toolbar).
5. Click, using the left mouse button, on every egg within the egg mass until all have markers. The total number of clicks will tally in the counter window.
6. Navigate to the next egg mass, click on a new counter and follow step 5 for all egg masses.
7. Counting is performed from left to right, prioritising top to bottom and counts are manually recorded in an Excel spreadsheet.
8. When all replicates are counted, six numbers are randomly selected for Quality Control and counted by another analyst. A 5% error rate is accepted.
9. Eggs in damaged or deformed egg masses are counted if the entire egg is visible. If eggs are not visible they are not included in the count.