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Ecotoxicological testing protocols for Australian tropical freshwater ecosystems

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Executive summary

Methodologies and data for deriving water quality standards for ecosystem protection have improved in recent years following the revision of the Australian Water Quality Guidelines (ANZECC & ARMCANZ 2000). The compilation of protocols in this report describes the ecotoxicological control measures developed for use to ensure protection of tropical freshwater ecosystems of northern Australia, with particular reference to the Alligator Rivers Region, Northern Territory.

The approach adopted by ***eriss*** was to develop, initially, water quality standards for the Alligator Rivers Region (ARR) based on chemical constituents. Then, recognising the deficiencies of control measures based solely on chemistry, to develop a set of ecotoxicity tests using local site-specific organisms within the ARR that could be used to (i) revise, in limited cases, these standards on the basis of local ecotoxicological data, and (ii) specify the dilution of waste water required to protect aquatic organisms. This approach is still relevant today, the procedures being used to revise standards and being applied to the control of water releases from ERA Ranger Mine NT, and also to assess effects and risks of chemical contaminants to tropical freshwater ecosystems.

This compilation of the ecotoxicological testing protocols developed by ***eriss*** for use in the Wet Dry tropics of northern Australia describes historical and recent references to all of the research testing protocols from the past 25 years. Accompanying each protocol is an appendix describing the specific species and culturing/husbandry requirements that have been developed. In addition, comprehensive datasheets are listed for each protocol to provide all the essential information to carry out high quality ecotoxicological testing.

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Introduction

Development of ecotoxicity tests

The use of local aquatic species for toxicological assessment of chemical contaminant impacts at particular sites is an approach that has been promoted and used in increasing industrial situations in Australia (ANZECC & ARMCANZ 2000, van Dam & Chapman 2001). This approach was adopted as part of the environmental protection program for ERA Ranger Mine in the mid 1980s and the rationale as well as history of ecotoxicity developments at ***eriss*** were extensively reviewed by Johnston (1994). Toxicity tests for 14 local species have been developed since this period with five local aquatic species regularly used for toxicity testing purposes (table 1). With the exception of the fish sac-fry survival test and cladoceran immobilisation test, all the test endpoints represent chronic responses. These and other species have been used to determine water quality guideline trigger values and waste water release dilutions, as described below.

Table 1 Current suite of site-specific toxicity tests using species local to the Alligator Rivers Region

Test organism	Duration (acute/chronic)	Test endpoint	Key reference
Chlorella sp. (green alga)	72 h (chronic)	Cell division rate	Franklin et al (1998)
Lemna aequinoctialis Welwitsch (duckweed)	96 h (chronic)	Growth (frond number)	This report
Moinodaphnia macleayi King (water flea)	3 brood (chronic)	Reproduction	Hyne et al (1996)
Moinodaphnia macleayi King (water flea)	48 h (acute)	Immobilisation	OECD (1999)
Hydra viridissima Pallas (green hydra)	96 h (chronic)	Population growth	Markich & Camilleri (1997)
Mogurnda mogurnda Richardson (purple-spotted gudgeon)	96 h (acute)	Larval sac fry survival	Markich & Camilleri (1997)

The ***eriss*** ecotoxicity laboratory was registered by the National Association of Testing Authorities (NATA) for 4 years (1991–94) with the BTT-C *Mogurnda mogurnda*, BTT-B *Hydra viridissima* and BTT-A and D *Moinodaphnia macleayi* tests accepted as fully registered tests (see table 2). (***eriss*** was the first laboratory in Australia to be registered for such tests.) ***eriss*** no longer maintains NATA registration, however, all the critical QA/QC regulations put in place during NATA registration have been retained by the laboratory, and it continues to operate to a very high standard.

The preferred approach to deriving receiving water standards is use of local ecotoxicological effects data. Early derivation of standards based upon local toxicity data focused on data acquired for uranium (ARRRI 1985). At the time this work was undertaken, the best available approach to trigger value derivation was the assessment factor method, wherein an assessment factor is applied to the species with the lowest ecotoxicity NOEC (no observed effect concentration) value. The new water quality guidelines (ANZECC & ARMCANZ 2000) classify trigger values derived using the assessment factor method as 'low reliability'. Rather, 'high reliability' values are preferred, especially where the ecosystem is considered highly valued, as is the case with the World Heritage listed Kakadu National Park, and for this a new

statistical distribution method is used whereby ecotoxicity data are fitted to a distribution curve to protect a chosen percentage of species; for sites of high conservation value, 99% of species is recommended.

In order to derive a 'high reliability', site-specific (Alligator Rivers Region) trigger value for uranium, chronic toxicity data for at least five local species from at least four taxonomic groups were required (ie stipulation for valid application of the statistical distribution method). Such data have been acquired gradually over the period ~1990 to the present. Trigger values are subject to change as further species are assessed and the methodology refined. A new uranium trigger value including recently acquired *Chlorella* sp data is currently being derived.

Summary of ecotoxicological test protocols developed at *eriss* for Australian freshwater tropical biota

The purpose of the following table (table 2) is to summarise and reference the protocols developed at ***eriss*** over the past 25 years. This research has been carried out using tropical freshwater organisms local to Magela Creek, Northern Territory, as a regulatory requirement for the protection of World Heritage Area Kakadu National Park from the adverse effects of mining activities. The tests identified are those currently routinely used in the laboratory and in the field *in situ*, tests under development, and tests that have been developed but are not used routinely. 'BTT' stands for Biological Toxicity Test and the letter after corresponds to each species and the test type that has been developed. If a test has been modified significantly from an existing protocol, it has been given a new identification, and the old test retained as archival information.

Table 2 Summary of ecotoxicological test protocols developed at ***eriss*** for Australian freshwater tropical biota

BTT code	Species	Test Description	Test Duration	Reference
A[2]	*Moinodaphnia macleayi* King	Cladoceran survival test	5–6 d	Hyne et al 1991 Hyne et al 1996
B[1]	*Hydra viridissima* Pallas	Hydra population growth test	96 h	Allison & Holdway 1988, 1989 Allison et al 1991 Hyne et al 1996 Markich & Camilleri 1997
C[2]	*Mogurnda mogurnda* Richardson	Gudgeon embryo–larval survival test	8 d	Holdway & Wiecek 1989 Holdway et al 1991 Hyne et al 1991 Hyne et al 1996
D[1]	*Moinodaphnia macleayi*	Cladoceran reproduction test	5–6 d	Hyne et al 1996 McBride et al 1988 McBride et al 1991
E[1]	*Mogurnda mogurnda*	Gudgeon sac-fry survival test	96 h	Markich & Camilleri 1997
F[3, 5]	*Melanotaenia nigrans* Richardson	Rainbowfish sac-fry survival test	96 h	Boyden et al 1995 Williams et al 1998
G[1]	*Chlorella* sp	Algal growth inhibition test	72 h	Franklin et al 1998
H[4]	*Moinodaphnia macleayi*	Cladoceran rapid response feeding test	20 h	Orchard 2000 Orchard et al 2002 Smith 2001
I[1]	*Moinodaphnia macleayi*	Acute cladoceran immobilisation	48 h	OECD 1999 Semaan 2000 Semaan et al 2001
J[4]	*Chironomus crassiforceps* Kieffer	Acute LC_{50} toxicity test	72 h	Peck 2000
		Sediment toxicity test	5 d	Peck et al 2002
L[1]	*Lemna aequinoctialis* Welwitsch	Lemna growth inhibition test	96 h	This report
			14 d	Allison & Holdway 1988
P[4]	*Azolla pinnata* R Brown	Azolla growth inhibition test	14 d	Brown 1994
S[3, 5]	*Amerianna cumingi* Adams	Snail egg production test	96 h	Humphrey et al 1995 Jones 1992 Lewis 1992 Suggit 1992
M[4]	*Ceratophyllum demersum* Linnaeus	Hornwort growth inhibition test	96 h	Markich et al (in prep)
IV[2]	*Ambassis macleayi* Castelnau	Juvenile perchlet test	14 d	Holdway & Wiecek 1988

1 Toxicity tests used routinely

2 Toxicity test protocols that have been archived after modification of methods

3 Toxicity tests under development and to be assessed for suitability

4 Toxicity tests developed but not used routinely

5 Toxicity tests used in the field *in situ,* and in the laboratory

Glossary of frequently used terms

Table 3 provides definitions of frequently used terms throughout this report (ANZECC & ARMCANZ 2000).

Table 3 Terms frequently used throughout the report

Term	Definition
BTT	Biological Toxicity Test – used to evaluate the relative potency of a chemical or water by comparing its effect on a living organism with the effect on a standard control preparation on the same type organism
Toxicant	A chemical capable of producing an adverse response (effect) in a biological system at concentrations that might be encountered in the environment, seriously injuring structure or function or producing death. Examples include pesticides/herbicides, heavy metals and biotoxins.
NOEC	No Observed Effect Concentration – the highest concentration of a toxicant that has no statistically adverse effect on the exposed test organism when compared to controls
LOEC	Lowest Observed Effect Concentration – the lowest concentration of a toxicant that has a statistically significant effect on the exposed test organism when compared to controls
BEC_{10}	10% bounded effect concentration, an alternative statistical measurement to the NOEC
MDEC	Minimal detectable effect concentration, an alternative statistical measurement to the LOEC
EC_{50}	The concentration of a toxicant that results in a 50% decline in the response of the test organisms
LC_{50}	The concentration of a toxicant which is lethal to 50% of the test organisms as a result of exposure over the test period
Concentration–response curve	A curve describing the relationship between toxicant concentration and percentage response of the test organism
Whole Effluent	A complex waste water (eg industrial discharge, sewage) which may be discharged into the environment
Trigger values	The approach used in this report involves fitting the most appropriate distribution from the Burr type III family of distributions to all NOEC data for a toxicant, to derive an estimated concentration that should protect at least χ% of the species in the environment. The value determined for appropriate level of protection of an aquatic ecosystem, must not to be exceeded through discharge of waste water.
Acute toxicity	Rapid adverse effect (eg death) caused by a substance in a living organism. Can be used to define either the exposure or the response to an exposure (effect)
Chronic	Lingering or continuing for a long time; often from periods of several weeks to several years. Can be used to define either the exposure of an aquatic species or its response to an exposure (effect). Chronic exposure often affects a life stage, eg reproduction, population growth etc.
Sublethal	Involving a stimulus below the level that causes death
Range-finding test	A BTT conducted to estimate a broad range of concentrations to be used for a definitive test, using a geometric series of concentrations
Definitive test	A BTT conducted to evaluate the effect of a toxicant over a narrow range of chemical concentrations, usually arithmetically determined

Preparation for toxicity tests

1 Deciding which test to run

Based on the purpose and objectives of, restrictions surrounding, and additional information relevant to the project, the research scientist overseeing the project will decide which species will be tested and the number and frequency of the tests. In most cases, a project will involve all or several of the five species listed in table 1, specifically: protocols BTT-B, BTT-E, BTT-G, BTT-L and BTT-D. Where other non-routine species or protocols are selected, additional Quality Assurance/Quality Control measures may need to be considered.

The research scientist will also make decisions on the concentrations tested and other specific details relating to the design of the test (if it deviates from the accepted BTT). The technician in charge of carrying out the test should obtain these details from the research scientist up to three days prior to the expected commencement date of the test.

2 Equipment

Refer to the relevant BTT.

3 Organisms

3.1 *Hydra viridissima* Pallas

Refer to BTT-B.

3.2 *Moinodaphnia macleayi* King

Refer to BTT-D, BTT-I.

3.3 *Chlorella* sp.

Refer to BTT-G.

3.4 *Lemna aequinoctialis* Welwitsch

Refer to BTT-L.

3.5 *Mogurnda mogurnda* Richardson

Refer to BTT-E.

3.6 *Ceratophyllum demersum* L

Refer to BTT-M.

4 Water

Refer to diluent, stock and test solutions in BTT methods.

4.1 Checklist for water preparation

Filtering:

NOTE: Use designated flasks, beakers, filter paper (eg Whatman No 42 2.5µm pore size), funnels etc for dilution / contaminant water.

1. Wash hands and arms.
2. Use one beaker for unfiltered water and one for filtered water, labelled appropriately.
3. Rinse flask, funnel, containers, measuring equipment and bucket with Milli-Q water after filtering. All objects which come in contact with contaminant water must be acid-washed (immersed in 10% nitric acid for 24 h then rinsed with Milli-Q water).
4. Keep diluent and test equipment separate and well distinguished.
5. Wash hands when filtering is finished.
6. When filtering is complete wipe down bench thoroughly with Milli-Q water, and wash all dirty equipment. This step should be done as soon as possible after the filtering.

Dispensing the water:

1. Ensure water is at 27 ± 1°C. Water can be allowed to equilibrate to this temperature inside temperature controlled incubators.
2. Wash hands and wipe down bench.
3. Start with the lowest concentration first ie Control.
4. Shake the test solution container to mix the solution.
5. If using the auto-dispenser, place in the test solution container and pump it at least twice to rinse it.
6. Rinse the bottle, vial etc with a little of the solution.
7. Add the correct amount of solution to designated containers for the experiment, chemical analysis, water quality parameters etc.
8. After dispensing water for one concentration, allow the auto-dispenser to drain before inserting into the next highest concentration container, then pump twice through to rinse it before beginning to dispense.
9. Return containers to the fridge (4°C).
10. Clean the auto-dispenser by pumping through 1% nitric acid about three times, draining, then pumping through demineralised water about six times, draining, and storing appropriately.
11. Wipe over the bench with Milli-Q water, and dry off. If test solution has been spilled wipe the bench over with 1% nitric acid, rinse well and dry.
12. Wash hands when finished.

NB: Dispense the test water several hours before the test and leave the containers covered in the incubator to reach the correct temperature.

5 Identification and marking of test items

5.1 Test number

Before the commencement of a toxicity test, a unique number is allocated for that test. This number is obtained from the Experiment Log. The number is accompanied by a letter to indicate which BTT is being followed (eg 157D). This identification is then recorded on each page of the test record data sheets and on each container used for the storage of the test waters.

5.2 Designation of code

A **code name** (usually determined by the research scientist overseeing the test) is allocated to each test and is entered in the Experiment Log (eg RP4-1 – where '1' indicates that it is the first test of a series). This code is recorded at the designated place on the data record sheets.

5.3 Labels

All test containers are labelled as follows:

Containers storing test solutions	Code name, test number, date of test commencement, treatment concentration
Test containers with animals	Replicate number, treatment concentration
Water parameter containers	Treatment concentration, fresh/old
Chemical analysis containers	Code name, test number, date of test commencement, treatment concentration, % acidification and if spiked

5.4 Colour code

Permanent marker pen colours are used to denote the test species:

Cladoceran tests	Blue
Green hydra tests	Green
Gudgeon larval tests	Red
Algal tests	Black
Lemna tests	Purple

Equipment and data sheets for each species are labelled with the relevant colour as a visual cue when testing with a combination of bioassays.

6 Recording of test data

All toxicity tests must be performed according to the methods detailed in the protocols. Results and observations are recorded on data record sheets, and must be signed and dated by the observer. Liquid paper is not permitted to change errors. These must be neatly crossed out, and the correct entry placed adjacent. If data are incorrectly recorded on a datsheet and must be re-written, the original datasheet must be retained in the test folder as a record.

7 Random number generation

7.1 Explanation

The position of the test animals in the incubator during a test must be a random selection. This is achieved by generating a set of numbers corresponding to each position, but in a random order.

For example, the hydra are placed in 18 petri dishes (three replicates for each of six concentrations), which are then placed on clear perspex trays with positions 1 to 18 marked. Each day a set of numbers 1 to 18 is randomised. The first number in the set is the position for Control Rep 1, the second number is the position for Control Rep 2, ..., the 18th number is the position for replicate 3 of the highest test concentration.

The following table is a typical random number series for a 96 h hydra test.

Treatment	Replicate		Day 1	Day 2	Day 3	Day 4
Control	1	R	18	12	9	16
Control	2	A	1	4	14	15
Control	3	N	13	16	10	18
1 ppb U	1	D	15	7	13	13
1 ppb U	2	O	9	15	18	10
1 ppb U	3	M	12	11	4	17
2.5 ppb U	1		17	14	12	6
2.5 ppb U	2	N	11	17	11	12
2.5 ppb U	3	U	14	2	7	4
5 ppb U	1	M	5	6	6	2
5 ppb U	2	B	4	3	8	7
5 ppb U	3	E	6	9	16	11
10 ppb U	1	R	10	10	15	14
10 ppb U	2	S	3	18	3	9
10 ppb U	3	|	8	1	1	8
20 ppb U	1	|	2	5	17	5
20 ppb U	2	V	16	13	5	1
20 ppb U	3		7	8	2	3

7.2 Generation

Random numbers are generated for each toxicity test. A new and unique set of random numbers is generated for each part of the test requiring randomisation.

8 Temperature considerations

Toxicity tests at ***eriss*** ecotoxicology laboratory are routinely performed at 27 ± 1°C for fish, hydra and cladocerans, and 29 ± 1°C for the lemna and alga. These temperature ranges reflect a mean natural environmental range for the species. To ensure that the temperature of the test containers remain at this temperature during observations and water changes, the following procedures are in place.

8.1 Fresh water

Before a water change commences, the temperature of the fresh water is measured to ensure it is at 27 ± 1°C. This is achieved by dispensing it early and leaving it in the incubator to equilibrate before use.

8.2 Observations

The test trays are placed on warming trays next to the microscopes for observation. These warming trays have been calibrated to 27 ± 1°C and the setting is marked on the side. These calibrations are checked every six months.

8.3 Before and after the observations

The test containers are left out of the incubator for the minimum possible time. For example, in BTT-D, Cladocera Reproduction test, adults are transferred to fresh water, randomised and returned to the incubator *before* neonates are counted.

8.4 Observers

Where possible, two observers work simultaneously to ensure that test containers are only out of the incubator for a minimum time. If only one observer is available, then unobserved trays should be kept in the incubator until ready to be observed.

8.5 Incubators/fridges

Log books are kept for recording daily temperatures of incubators and solution storage fridges.

9 Error recognition

All workers involved in toxicity tests should be aware of the expected results, and hence be able to recognise unusual results. This may then lead to the need for corrective action to be taken on the spot (eg re-measuring of pH, conductivity, dissolved oxygen if a discrepancy is observed).

9.1 Results of toxicity tests

The USEPA recommended flow chart (fig 1) identifies the steps taken to analyse data for point estimation (eg EC_{50}) or hypothesis testing (NOEC and LOEC). Both of these analyses can be done sequentially using the ToxCalc™ (ToxCalc™1996) program. A concentration-response curve visually depicts the results to assist in choosing the appropriate statistical method.

An expected LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) can be estimated from previous test results. When a new test is first performed, this will not be possible (eg a range finding test for a new chemical or whole effluent).

It is the responsibility of the research scientist and test supervisor to make observers aware of these expected results and when trends might become apparent in the test. The observers should report immediately to the supervisor if results obtained differ from expected. The supervisor should also regularly check observed results throughout the test for unusual results and observations.

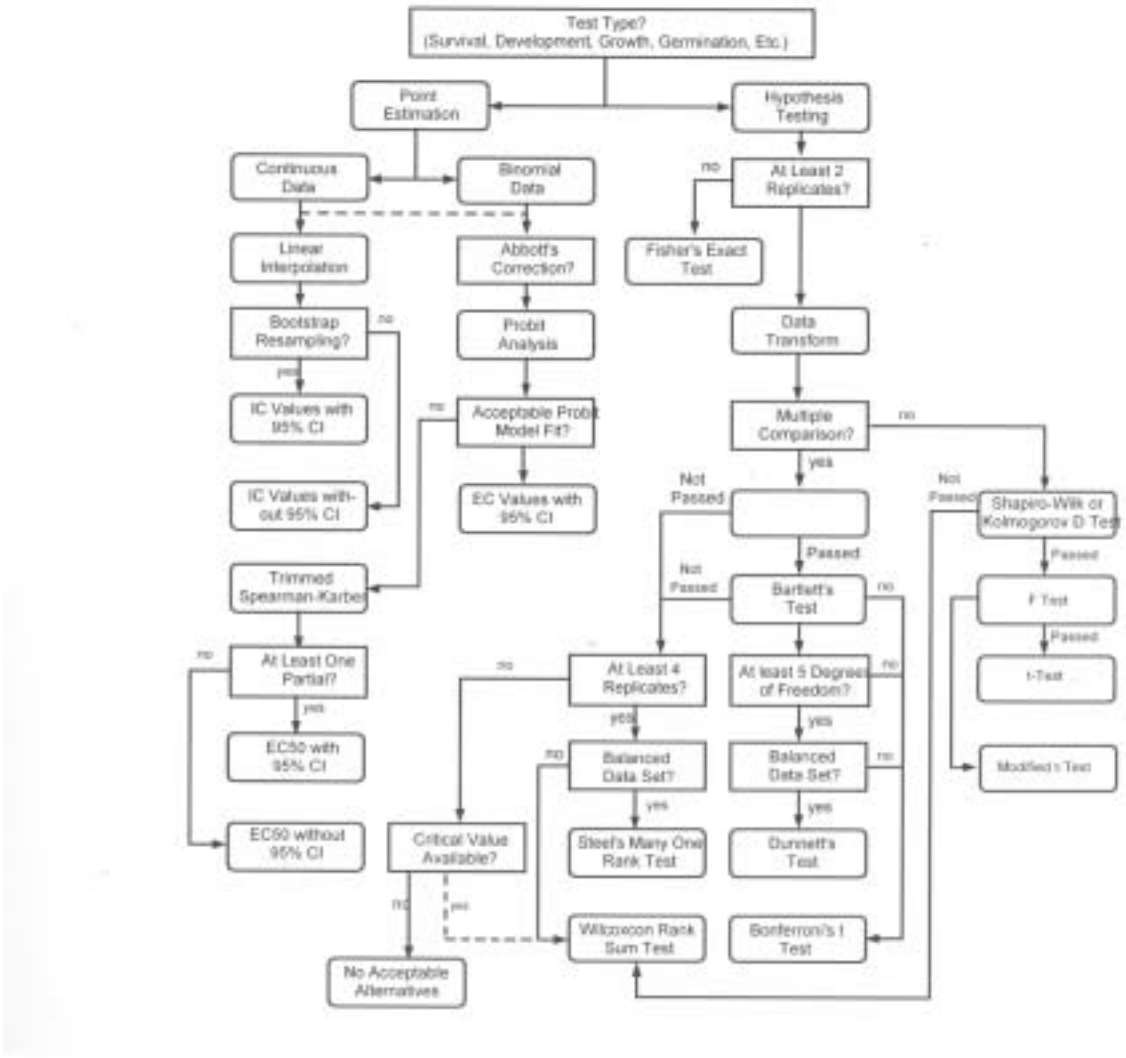


Figure 1 USEPA recommended flow chart

If unexpected results are observed, it is the decision of the supervisor to determine if any action is to be taken.

Examples of action taken could be:

1. Check pHs, conductivities, DO, etc.
2. Abort test
3. Continue test
4. Restart test

Any such action should be described on the test data sheets.

9.2 Water parameter measurements

Accepted limits across data of each group:

pH – within ± 0.2 units of the values obtained on Day 0.

Conductivity – within ± 10% of the values obtained on Day 0.

Dissolved oxygen - greater than 70% of the air saturation value throughout the test at a temperature of 27°C.

Note: extreme pH and conductivity values may be important characteristics of waste waters, and any outliers/anomalies must be immediately reported to the research scientist/supervisor.

Corrective action

If any readings do not lie within acceptable limits, the sample should be re-measured.

If the readings are still not within acceptable limits, the research scientist/supervisor must be informed.

The supervisor will then determine if any action will be taken.

Such action could be:

1. Re-calibrate the meters
2. Observe the technician's procedure
3. Check on sample collection/preparation procedure
4. Check test organism observations and results
5. Check stock cultures of organism to determine normal health in culture

If the water quality parameters are marginally out of the acceptable limits, no action may be necessary, but this would be noted on the test reports.

Any action taken (including re-measuring of samples) should be described on test data sheets.

9.3 Acceptability of test data

For validity requirements refer to each BTT protocol.

BTT-B: Green hydra population growth test

Hydra viridissima Pallas showing developing bud

BTT-B: Green hydra population growth test

B.1 Objective

The objective of a test series (ie 3–4 definitive tests) is to determine the concentrations of a specified chemical or whole effluent that shows:

a) The No Observed Effect Concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the 10% Bounded Effect Concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk 1993);

b) The Lowest Observed Effect Concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response becomes significantly ($P \leq 0.05$) lower than that of the control (Ahsanullah & Williams 1991);

c) The median effect concentration (EC_{50}), is the concentration of chemical in solution that is estimated to be effective in producing a sublethal response in 50% of test organisms. This is measured as the 50% reduction on the population growth of test hydra (*Hydra viridissima*) over 96 h.

B.2 Principle of the test

Asexually reproducing (budding) test hydra are exposed to a range of chemical concentrations for 96 h. Observations of any changes to the hydra population (ie 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).

B.3 Test organism

The species is *Hydra viridissima* Pallas (Cnidaria, Hydrozoa). *H. viridissima* is referred to as 'green' hydra because of its green colouration resulting from the presence of a symbiotic green alga in the gastrodermal cells of the animal. Although the precise distribution of this species has not been mapped, it has been found in a variety of aquatic habitats in northern Australia. Test hydra were obtained from laboratory cultures as described in Appendix B. Test organisms are selected on the basis that the hydroid is bearing one tentacled bud. Asexual budding is a characteristic of hydra in optimal environmental conditions. Hydra selected for testing must be free of overt disease and gross morphological deformity (ie show no signs of clubbing or contraction). Source of the test hydra is from Magela Creek.

B.4 Dilution water

There are two diluent water types which are routinely used. In addition, dechlorinated filtered Darwin tap water can be used as an analogue of low conductivity, low mineralised soft water. The aim of the test will determine which water type is used.

B.4.1 Synthetic water

'Synthetic' water simulates the inorganic composition of Magela Creek water during the Wet season. Magela Creek water is very soft, slightly acidic and has a low buffering and complexation capacity. These qualities are predicted to maximise the toxic response of an organism, and hence, provide the greatest probability of detriment to organisms exposed to metals. The ionic composition of Magela Creek water is representative of sandy braided streams throughout much of the Wet-Dry tropics. The synthetic water is prepared by adding analytical grade reagents to Milli-Q water (< 1 µS cm^{-1}) (table B.1) in acid-washed polyethylene containers, as close as practical to the start of the test (see appendix A.B.5). The pH of the test water is adjusted to the required level (in this case 6.0 ± 0.15 at 27 ± 1°C) with 0.02 M HNO_3 or 0.0125 M NaOH. The test water is stored in sealed polyethylene containers and refrigerated (4°C) until use.

Table B.1 Mean Nominal Composition of the synthetic water

Physico-chemical parameter	Total concentration
pH	6.0 ± 0.15[a]
Temperature (°C)	27 ± 1[a]
Na	1.00 mg L^{-1}
K	0.37 mg L^{-1}
Ca	0.45 mg L^{-1}
Mg	0.60 mg L^{-1}
Cl	2.32 mg L^{-1}
SO_4	3.12 mg L^{-1}
HCO_3	2.63 mg L^{-1}
NO_3	0.07 µg L^{-1}
Fe	100 µg L^{-1}
Al	70 µg L^{-1}
Mn	9.7 µg L^{-1}
U	0.10 µg L^{-1}
Cu	0.70 µg L^{-1}
Zn	0.70 µg L^{-1}
Pb	0.12 µg L^{-1}

a pH and temperature were tightly regulated as described in the text

B.4.2 Natural stream water

Natural stream water is the receiving water taken upstream of the waste water discharge outlet. In the Wet season water is collected from Magela Creek upstream of Georgetown pumping station (latitude 12° 40' 28", longitude 132° 55' 52) or from Bowerbird Billabong (latitude 12° 46' 15", longitude 133° 02' 20") in the Dry season. It should be collected in acid-washed plastic containers as close as is practicable to the start of the test, ie afternoon prior to test commencement, and stored at 4°C overnight. Within 48 hours of collection, the required amount of water for the test should be filtered through a fine pore size filter (eg Whatman No 42, 2.5 µm) capable of removing 'wild' zooplankton. The water should be stored in covered polyethylene containers at 4°C for a maximum period of three weeks to avoid any seasonal variation in water quality and deterioration of water quality in storage

(Magela Creek water is poorly buffered and therefore is not stored for long periods). Other natural water types may have different properties and may be held for longer periods – this would be determined for each natural water type.

B.4.3 Filtered Darwin tap water

Darwin tap water is low conductivity, low mineralised good quality soft water. At the ***eriss*** laboratory, water travels from the mains supply predominantly through PVC pipe to two activated carbon filter units. After passing through these the water is then pumped into the roof cavity and is held in two temperature controlled (27 ± 1°C) holding tanks. When water is required for use in culturing or as diluent water for testing, it travels through insulated PVC plastic pipes from the holding tanks to the aquaculture laboratory. General water quality parameters are routinely measured, while water chemistry samples are regularly collected for analysis of major anions and cations.

B.5 Stock solutions

B.5.1 Chemical solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in an acid-washed, polyethylene container and refrigerated (4^oC). The source of the stock solution (eg date of preparation, by whom), is marked on the bottle and on an information sheet. Immediately prior to use for test water preparation the stock solution is allowed to equilibrate to room temperature.

B.5.2 Whole effluent solutions

Whole effluent stock solutions are collected from the designated site as close as is practicable to the start of the test, in an acid-washed plastic or glass container (depending on the chemical properties of the effluent). The sample should be kept sealed, clearly labelled and refrigerated at 4°C until required for test commencement.

B.6 Test solutions

Test solutions are prepared by diluting a stock solution with diluent water. The pH is then adjusted if necessary, using 0.02 M HNO_3 or 0.0125 M NaOH. Test solution concentrations are determined from the results of range-finding studies. Test solutions are prepared in bulk at the start of a test in 2–5 L polyethylene or glass screw-topped containers and refrigerated (4^oC) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies significantly when stored for the test period.

B.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the hydra are placed, or the hydra themselves, should be chemically inert.

B.7.1 Container preparation

All containers (ie bottles, Petri dishes and lids etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 μS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least 3 times with Millipore water (< 1 μS cm^{-1});
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with appropriate diluent or control water. Other equipment should be rinsed thoroughly with Milli-Q water (< 1 μS cm^{-1}) before use.

B.7.2 Temperature control

Tests are conducted at 27 ± 1°C using a constant temperature incubator. When removed from the incubator for observation, the temperature of the test containers are maintained at 27 ± 1°C by the use of warming trays on the microscope bench.

B.7.3 Photoperiod control

Tests are conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie 30–100 μmol m^{-2} s^{-1}).

B.7.4 Equipment

- Light – tight constant temperature incubator
- Milli-Q water purification system or equivalent
- A-grade glass volumetric flasks
- Chemicals and reagents
- Analytical balance and weigh boats
- Filter apparatus – 5 L plastic Buchner funnel, 5 L glass flask with side arm, vacuum pump and tubing
- Membrane filters – 2.5 μm pore size (Whatman No 42)
- Magnetic stirrer and stirrer bars
- 5 L polyethylene containers (to hold treatment solutions, one per solution)
- Refrigerator for storage of test and stock solutions
- 90 mm diameter disposable plastic Petri dishes with lids (3 replicates per solution)
- 150 mL tall-form polypropylene containers (for water parameter measurement – one per solution)

- 45 mL plastic vials (eg Nylex type 12, for aliquoting 30 mL to respective treatment Petri dishes – 3 replicates per solution)
- pH meter, electrical conductivity EC meter and dissolved oxygen meter
- Binocular dissecting microscope with bright field/dark field illumination
- Automatic 0–50 mL dispenser
- Three clear perspex trays, each capable of holding Petri dishes, with position numbers 1 to 24 marked (extra trays can be added if more test solutions are run in a test)
- Laboratory warming trays, set at 27^{o}C, capable of accommodating the clear plastic trays
- Random number generator
- Two plastic trays, one of such a size to hold sixteen 45 mL vials and the other to hold twenty-four 45 mL vials
- Pasteur pipettes, with internal tip diameter of ~ 1 mm
- Data sheets
- 50–200 mL acid-washed bottles (glass or polyethylene depending on toxicant) with plastic screw caps, for analytical chemistry samples.
- Brine shrimp hatchery (Appendix B.4)

B.8 Test environment

The preparation and storage of test solutions, culturing of test hydra, and conducting tests should be carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

B.9 Data recording

Test animals are observed and data recorded at 24 h intervals after the commencement of the test (when t = 0 h). Observations made at the end of the first 24 h period are designated as Day 1 observations; at the end of the second 24 h period, Day 2 observations etc. Water quality parameters are measured and adjusted (where appropriate) and recorded at the beginning and end of each 24 h period, and are designated as Fresh Water Day 1, 24 h -old Water Day 1, respectively, and so forth during the test.

B.10 Test procedure

Day 0 (ie starting day of the test t = 0 h)

1. Prepare the test solutions (as outlined in Section B.4) and leave at room temperature.
2. Isolate enough suitable hydra (eg at least 260 for an eight concentration test) into diluent water into three Petri dishes and allow to equilibrate to room temperature. A ‘suitable test hydra’ is a hydra with one developing bud. The bud must not be *fully* developed (ie tentacles are present only as ‘bumps’, and the bud must not appear ready to detach from the main stem of the hydroid).

3. Dispense 60 mL of each test concentration into the appropriately labelled (ie. 'New water A' etc) 150 mL plastic containers for water parameter analysis.
4. Dispense 30 mL aliquots of each test concentration (normally eight) into three appropriately labelled replicate Petri dishes (ie 3 x 30 mL for each test solution), and arrange in three replicate groups on clear plastic trays (eg. Control replicate 1 to X $\mu g\ L^{-1}$ on Tray 1).
5. Using a microscope and Pasteur pipette, pick out one hydra from the isolated stock and place into control replicate 1, without allowing the pipette tip to contact the test concentration.
6. Repeat for remaining test concentrations of replicate 1, working up in concentration, and ending with the highest concentration.
7. Discard the used pipette and select a new one if at any time it comes in contact with any of the test concentrations.
8. Repeat steps 5–7 until all test dishes for that replicate group contain ten hydra.
9. 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 (eg all buds must be attached). If damaged, replace immediately with 'suitable test hydra' using a new pipette.
10. Repeat steps 5–9 for the remaining two replicate groups.

Note: More than one person can distribute test hydra simultaneously, with the distribution appropriately split into replicate groups.

11. Cover the dishes and place them in the random order for that day (Section B. 11), in the positions 1 to 24.
12. Place trays in the incubator.

Completion of this stage constitutes the start of the test (time = 0 h).

Note: Whenever test dishes are removed from the incubator maintain them at 27^{o}C (eg by placing them on a warming tray).

13. Measure water quality parameters ie pH, conductivity and dissolved oxygen.
14. At t = 2 h (after test commencement) observe each Petri dish under the microscope and record the following:
    a) Counting and recording the number of hydroids, with or without buds;
    b) Noting if tentacles appear clubbed or contracted; and
    c) Noting any other observations that suggest the hydra are not behaving or developing normally eg floating, elongated etc.

Note: Observations are recorded at t = 2 h on the data sheets. To avoid observer bias, select a different replicate to observe each day. Also each day, commence observations with the next highest chemical concentration to that observed on the previous day (Section B.12). Do not change positions of the dishes on the tray and return dishes immediately to the incubator afterward:

Note: Water movement will cause temporary tentacle contraction, therefore allow the water to settle before recording observations.

Day 1

15. Dispense 70 mL of the test solutions into appropriately labelled 150 mL vials for water parameter measurement.
16. Dispense 3 x 35 mL of each test solution into appropriately labelled 45 mL vials. Cover dispensed solutions and allow them to equilibrate to 27^{o}C in an incubator.
17. Twenty hours after the commencement of the test, remove the trays from the incubator, arrange the test dishes into replicate groups, observe under the microscope and record as Day 1 observations.
18. After recording observations for a particular dish, feed each hydra in the dish individually.

Each hydra is fed at least 3–4 live brine shrimp nauplii, *Artemia franciscana* (Appendix B.4). The nauplii are rinsed and suspended in control water and placed in each dish using a glass Pasteur pipette. Feeding is allowed to proceed *ad libitum* for 4 hours

19. After all hydra have been observed and fed in the dishes, place the test dishes onto trays in the random order for the day (Section B.11), and return the trays to the appropriate position in the incubator.
20. Twenty-four hours after the commencement of the test, 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 second Petri dish (or cleaning dish);
    c) An aliquot of the test solution (5 mL) is immediately added to cover the bottom of the test dish, the swirling process is repeated, and the solution tipped into the cleaning dish;
    d) The remaining fresh solution (30 mL) is immediately added to the test dish;
    e) Any hydra that are dislodged into the cleaning 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, with care taken to minimise removal of test solution;
    g) The cleaning dish is checked again for hydra, with any found being returned to the test dish; and
    h) The solution in the cleaning dish is collected for the measurement of water quality parameters in each treatment after 24 h, ie Day 1 ‘Old or 24 h’ water.

Note: To ensure that cross-contamination does not occur, obtain a new pipette and cleaning dish whenever a dish of lower chemical concentration is cleaned after a higher concentration.

21. Measure the physical water quality parameters (ie. pH, conductivity, dissolved oxygen) at the end of 24 h.

Day 2–3

22. Repeat steps 15–20 (ie 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).

23. Measure the physical water quality parameters of the ’24 h’ water and record for the appropriate day.

Note: On each day a new set of random numbers must be used for the position of each Petri dish in the incubator for the next 24 h period (Section B.11).

Day 4

24. Count and record observations for each test dish 96 h (4 x 24 h) after the start of the test. Do not feed hydra and do not renew test solutions.

25. Measure the physical water quality parameters and record as Day 4.

Test is complete.

B.11 Randomisation

Randomness is an important component of the experimental design. Random distribution of hydroids in test Petri dishes is achieved via steps 4–7. The Petri dishes are randomly assigned to positions on trays each day, meaning they will also have a random position in the incubator. Random numbers are obtained from a random number table or generator for each day of the test; a set of random numbers is not to be reused. When the hydra have to be observed, the Petri dishes can be sorted into replicate groups for greater convenience. This avoids the continual changing of glass pipettes by working through the water changes from a lower to a higher chemical concentration. At the end of the water changes the Petri dishes are again randomly placed on trays and returned to the incubator.

B.12 Avoiding bias

To avoid observer bias there should be at least two observers, however, in some instances this will not be possible. Each observer randomly selects a replicate group to record each day, and observations commence with the next highest chemical concentration to that which was first observed the previous day. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section B.13, should be used.

B.13 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Uranium (added as uranyl sulphate) is used in a concentration range from 20–150 ug/L. Synthetic water is used as diluent (appendix A.B.5). The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to uranium. If the value falls outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

B.14 Acceptability of test data

The test data are considered acceptable if:

1. The recorded temperature of the incubator remains within the prescribed limits;
2. More than 30 healthy hydroids remain in each control dishes at the end of the test period;
3. The recorded pH are within the prescribed limits (usually ± 0.2 unit of Day 0 values for each test concentration);
4. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 27^{o}C;
5. The conductivity for each test solution was within 10% of the values obtained on Day 0.
6. The result of reference toxicity testing is within the set limits.

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

B.15 Analysis of test data

The endpoints of the hydra population growth test are measured as the 96 h EC_{50}, the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC). The EC_{50} is the effective concentration giving 50% reduction in hydra population growth rate over 96 h compared to the controls. This is calculated using Trimmed Spearman-Karber analysis (Hamilton et al 1977) or Maximum Likelihood Probit analysis (ToxCalc™ Version 5.0.23D, Tidepool Software). After testing the data for normality and homogeneity of variances, Dunnett's Multiple Comparison Test is used to determine which treatments are significantly different from one another (normally, using an alpha (α) level of 0.05). This information enables estimation of the LOEC and NOEC. Alternative statistical measures to the NOEC and LOEC can also be calculated. The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, is estimated using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, is estimated using the approach described by Ahsanullah and Williams (1991).

Appendix B

BTT-B: Green hydra – Stock culture maintenance

A.B.1 Primary hydra stock culture

Green hydra (*Hydra viridissima*) are cultured in the laboratory in bubble-aerated filtered stream water, synthetic water or filtered Darwin tap water, held in 2 L glass bowls (primary stock). The bowls are loosely covered with clear polyethylene food film (eg. Glad®Wrap) to allow ventilation around the sides and protection from airborne dust and contaminants. The culture water is taken from the same batch of diluent water that is used to commence the test. The water movement caused by the gentle aeration causes most hydra to attach to the side of the bowl via the basal disc, thus reducing time taken to perform water changes.

Primary stock hydra are fed at regular intervals, three to four times a week. One week prior to commencing a test, the primary stock hydra are fed on a daily basis to achieve the maximum budding rate. Prior to commencement of this intensive feeding, hydra are observed and notes on culture health and density are recorded in the primary hydra stock log book. A sample of water is then taken and the dissolved oxygen (DO) concentration measured and recorded as a water quality check. Hydra are then fed with newly-hatched brine shrimp nauplii (*Artemia franciscana*; Appendix B.4). Cladocera (*Moinodaphnia macleayi*) are also fed at least once a week to the primary and secondary hydra cultures as a natural diet supplement (refer to protocol BTT-D cladoceran reproduction test, Appendix D1, for cladocera husbandry techniques). Prior to being fed to hydra, the brine shrimp are thoroughly washed and suspended in diluent water. They are then pipetted into each primary stock bowl so that they are evenly distributed over the hydra. The hydra are allowed to feed for at least 30 min, and up to 4–5 h when possible. At the end of the feeding period, uneaten brine shrimp and regurgitated food pellets are removed by performing a ‘scrub’ clean.

Scrub cleaning is performed three to four times per week, and is done as follows. After observations are made and recorded and samples taken to measure DO levels, the water is gently swirled around in each bowl and emptied into a cleaning dish (eg a white 4 L plastic ice-cream container). Diluent or culture water is added and the procedure repeated until each bowl is free of brine shrimp (if they have just been fed). A small amount of diluent water is poured into the bowl. The hydra are removed by using a finger to gently push the attached hydra away from the sides of the bowl. Clean hands, or hands covered by powder-free gloves should be used to perform this procedure. The water is again swirled around and poured into another cleaning dish, along with the dislodged hydra. The detached hydra are allowed to settle into a corner of the cleaning dish by slightly tipping the dish. Using a glass Pasteur pipette, the hydra can then be transferred to a clean glass bowl containing about 1.5 L of fresh water. It is important that the hydra are maintained at a density of 2 to 3 to ensure continued health (fig A.B.1). Bowls are regularly washed by analytical-grade dishwasher (eg Gallay Laboratory 999 Micro). Alternatively, the same bowl may be used continuously, as long as it is wiped clean each time with a lint-free cloth once the hydra are removed. Immediately prior to use, the bowls are rinsed with fresh culture or diluent water.

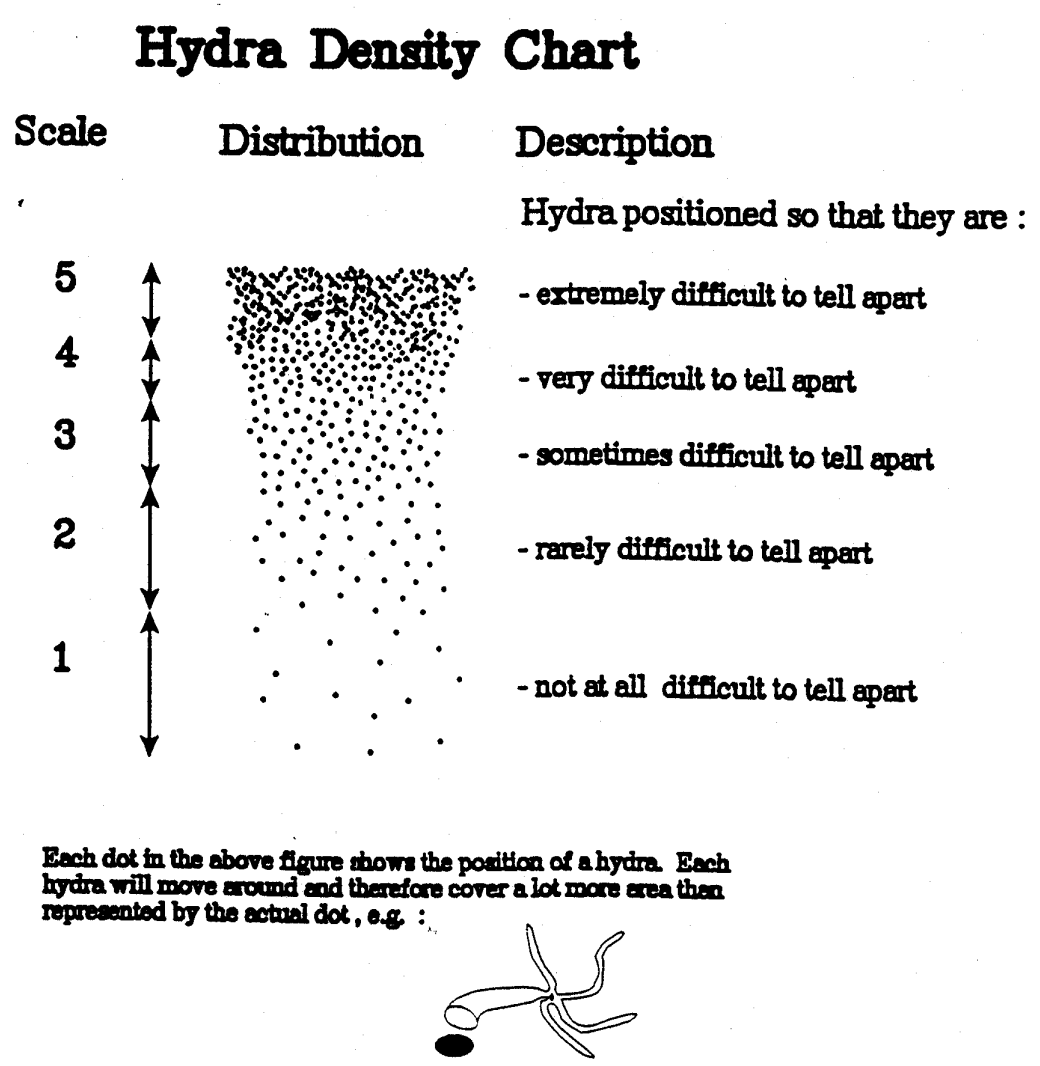


Figure A.B.1 Hydra density chart

A.B.2 Secondary hydra stock culture

A secondary stock of hydra are maintained in filtered tap-water filled aquaria in a separate location, as a precaution against contamination or accidents. The aquaria are maintained as 'community' aquaria, with small fish (eg. *Ambassis* sp., *Pseudomugil* sp.) and freshwater snails.

The secondary hydra stock are fed daily with brine shrimp and occasionally with excess cladocera (from primary or secondary cultures – usually ~ 100–200 cladocerans), and the aquaria cleaned as necessary. Excess hydra are gently pushed away from the sides of the aquaria and siphoned out. A third of the aquarium water should also be siphoned out and replaced as part of the cleaning process.

A.B.3 Sexual reproduction in hydra cultures

Periodically, hydra are observed to reproduce sexually, making it difficult to maintain an isogenic population. This could be related to fouling of the holding water and fungal growth on the uneaten brine shrimp. The frequency with which sexual reproduction occurs can

sometimes be reduced by increasing the rate of feeding and cleaning of the primary cultures. If fungal contamination is observed at any time, the bowls should be given a scrub clean. Sexual reproduction may also result from other factors such as too high a population density, in which case the population should be thinned.

A.B.4 Culturing of live brine shrimp larvae

A.B.4.1 Hatching brine shrimp larvae

Brine shrimp (*Artemia franciscana*) are used as food for many types of aquatic organisms, including larval fish and hydra. Brine shrimp can be cultured in a variety of containers to give an uninterrupted supply of nauplii (juvenile brine shrimp). The most appropriate type of culture containers are conical flasks (conical 1000 mL separation funnels are ideal) which, when inverted with the neck downwards, can be bubble-aerated from the bottom with oil-free compressed air. A 1000 mL salt solution is made by dissolving 30 g of uniodised coarse rock salt or sea salt, in 1000 mL of warm water (27°C). After the salt is fully dissolved, one teaspoon (~5 g) of commercially harvested, dried brine shrimp cysts are added. Gentle but constant bubbling from the bottom of the flask prevents eggs from settling. Brine shrimp eggs will hatch in 24–30 h at an incubation temperature of 27°C or in an outside shaded position. At lower temperatures, hatching is delayed. On cloudy days the culture may need to be directly illuminated by a fluorescent lamp, as hatching is also light dependent.

A.B.4.2 Harvesting brine shrimp larvae

To harvest the newly-hatched nauplii in a 27°C environment, the compressed air is turned off 24–30 h after adding the eggs, allowing the nauplii to settle and empty egg shells to float. After ~5 min, the nauplii are strained through a 250 µm fine nylon mesh net which is able to retain the nauplii. The nauplii are rinsed thoroughly with the test dilution water. The washed nauplii are then re-suspended in a small volume of dilution water (about 5 mL) and placed in a small beaker or Petri dish which is inclined at an angle of approximately 45° towards the light. Live nauplii will concentrate in the upper layer, while the unhatched cysts will remain on the bottom of the container. The upper layer, containing live nauplii, is then collected for feeding. A Pasteur pipette or syringe is used to collect and distribute the nauplii.

A.B.5 Preparation of synthetic water

1. Prepare the stock solutions (table A.B.1) in 1L volumetrics with Milli-Q water. Transfer to clean 1L plastic bottles and store at 4°C until required.

Note: these ingredients are stored at 4°C, and will require replacing at 18–24 month intervals.

2. Fill a 5 L volumetric flask with Milli-Q water and pour this into a clean 25 L plastic barrel designated for synthetic water preparation.
3. Add the appropriate amount of the 7 solutions (described below) to the partially filled 5 L volumetric flask. Make flask up to volume with Milli-Q and pour into the barrel.
4. Fill the 5 L flask twice more to make the volume in the barrel equal 20 L.
5. Aerate overnight to allow mixing and gaseous exchange.
6. Check pH after a minimum of 12 hours aeration and adjust pH to 6.0 ± 0.15 using 0.05 M H2SO4 or 0.05M NaOH.
7. The water can be stored at 4°C for up to 2 weeks if required. The pH is to be checked before use to ensure it remains within range.

Table A.B.1 Method for preparation of synthetic water

	Ingredient	Stock Solution (g/L)	Media Solution
1	$NaHCO_3$	72.34	1 mL/20 L
2	$Al_2(SO_4)_3.6H_2O$	17.26	1 mL/20 L
3	$MgSO_4.7H_2O$	121.52	1 mL/20 L
4	$CaCl_2.2H_2O$	32.96	1 mL/20 L
5	KCl	14.09	1 mL/20 L
6	$FeCl_3.6H_2O$	10	1 mL/20 L
7	Trace Element Solution	In 1 L add:	0.5 mL/20 L
	$CuSO_4.5H_2O$	0.11	
	$ZnSO_4.7H_2O$	0.123	
	$Pb(NO_3)_2$	0.008	
	$MnSO_4.H_2O$	1.188	
	$UO_2SO_4.3H_2O$	0.007	

PROTOCOL SHEET

Project Name **Project Number:**

Test Number: 611B **Test Name:** Hyd_Djalk_02 **Start Date:** 17/12/02

BTT: B-hydra population growth test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	3000 mL	0 mL
B	0.3%		9 mL
C	1.0%		30 mL
D	3.2%		96 mL
E	10%		300 mL
F	32%		960 mL
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: 611B **Test Name:** Hyd_Djalk_02

Toxicant: Djalkmara Billabong **Diluent:** Magela creekwater

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	As per lab manual	As per lab manual
Site	Djalkmara pump station	Georgetown
Transportation	22 L jerry cans	22 L jerry cans
Comments	Collected by XX	Collected by XX

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: Chemical weighed out by:

Balance used: Stock solution prepared by:

TEST DETAILS			
BTT	B		
Species:	*Hydra viridissima*		
Start Date:	17/12/02		
Start Time:	10:00am		
Started by:	XX		
Incubator no:	Beige-1		
Temps OK?	yes		
Test waters prepared by:	XX		
Supervisor:	XY		
Chem. analysis: **J/N,S/N & date** **Submitted by:**	NTEL - metals AGAL – TOC, alkalinity		
Departure from BTT / comments:			

FRESH water parameters

Test No: 611B	**Test:** Hyd_Djalk_02	**Species:** *Hydra viridissima*	
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong	
pH:	**Cond:**	**pH:**	**Cond:**

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**					**Observer / Date:**
0	pH											
	Cond											
	DO											
1	pH											
	Cond											
	DO											
2	pH											
	Cond											
	DO											
3	pH											
	Cond											
	DO											
4	pH											
	Cond											
	DO											
6	pH											
	Cond											
	DO											

QC: pH stays w/i ±0.2 unit of Day 0 values for each Conc.; Cond for each test soln is w/I 10% of Day 0 values; DO conc. >70% air saturation value for each conc.

24 hour OLD water parameters

Test No: 611B,E	**Test:** Hyd_Djalk_02	**Species:** *H. viridissima*
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**			**Observer/ Date:**
1	pH									
	Cond									
	DO									
2	pH									
	Cond									
	DO									
3	pH									
	Cond									
	DO									
4	pH									
	Cond									
	DO									
5	pH									
	Cond									
	DO									
6	pH									
	Cond									
	DO									

HYDRA POPULATION GROWTH

Test No.: 611B **Species:** *Hydra viridissima*

Test Name: Hyd_Djalk_02 **Start Date:** 17/12/2002 **Initial no./dish:** 10 **Replicate no.:** 1

Day	Concentration / Observation	A Control	B 0.3%	C 1.0%	D 3.2%	E 10%	F 32%			
2hr	No. of hydra									Observer/
	No. Normal									Date:
	No. fully contracted									
	Clubbing (Y/N)									
	Comments									Checked by:
1	No. of hydra									Observer/
	No. Normal									Date:
	No. fully contracted									
	Clubbing (Y/N)									
	Comments									Checked by:
2	No. of hydra									Observer/
	No. Normal									Date:
	No. fully contracted									
	Clubbing (Y/N)									
	Comments									Checked by:

HYDRA POPULATION GROWTH (continued)

Test No.: 611B **Species:** *Hydra viridissima*

Test Name: Hyd-Djalk_02 **Start Date:** 17/12/2002 **Initial no./dish:** 10 **Replicate no.:** 1

Day	Concentration / Observation	A Control	B 0.3%	C 1.0%	D 3.2%	E 10%	F 32%			
3	No. of hydra									Observer/
	No. Normal									Date:
	No. fully contracted									
	Clubbing (Y/N)									
	Comments									Checked by:
4	No. of hydra									Observer/
	No. Normal									Date:
	No. fully contracted									
	Clubbing (Y/N)									
	Comments									Checked by:

BTT-D: Cladoceran reproduction test

Moinodaphnia macleayi King showing juvenile at left, and adult female with eyed young in the brood pouch on the right

BTT-D: Cladoceran reproduction test

D.1 Objective

The objective of a test series (ie 3–4 definitive tests) is to determine the concentrations of a specified chemical or whole effluent that shows:

a) The No Observed Effect Concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the 10% Bounded Effect Concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk 1993);

b) The Lowest Observed Effect Concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response becomes significantly ($P \leq 0.05$) lower than that of the control (Ahsanullah & Williams 1991);

c) The median effect concentration (EC_{50}), is the concentration of chemical in solution that is estimated to be effective in producing a sublethal response in 50% of test organisms. This is measured as the test concentration causing a 50% reduction in the number of neonates produced.

D.2 Principle of the test

Asexually-reproducing female Cladocera that are less than six hours old (ie neonates) at the beginning of the test, are immersed in a range of concentrations of the test water to be assessed under 'static renewal' conditions. These 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, rather than assuming missing animals are dead. The test is terminated when three broods have been produced by each surviving control female (normally over a 5–6 day period). The method is based on the *Ceriodaphnia* Survival and Reproduction Test developed by the USEPA (Horning & Weber 1985) and was initially developed using a local species by McBride et al (1988).

D.3 Test organism

The test species is *Moinodaphnia macleayi* King (Crustacea, Cladocera) commonly known as a water-flea or cladoceran (Smirnov & Timms 1983). *M. macleayi* is a littoral/benthic species commonly found in weed bed habitat on the Magela Creek floodplain (Julli 1986). The test water fleas should be less than six hours old at the start of the test (ie neonates), and are obtained from laboratory stock cultures as described in Appendix D.1. All the test water fleas must be asexually-reproducing (parthenogenetic) females. Asexual reproduction is a characteristic of this type of organism under optimal environmental conditions. Test water fleas should be free from overt disease or gross morphological deformity. Original test cladocerans were collected from Magela Creek, with recent cultures restocked from Bowerbird Billabong in September 2002.

D.4 Dilution water

There are two diluent water types which are routinely used – natural stream water and dechlorinated filtered Darwin tap water (which can be used as an analogue of low conductivity, low mineralised soft water). The aim of the test will determine which water type is used.

D.4.1 Natural stream water

Natural stream water is the receiving water taken upstream of the waste water discharge outlet. In the Wet season water is collected from Magela Creek upstream of Georgetown pumping station (latitude 12° 40' 28", longitude 132° 55' 52") or from Bowerbird Billabong (latitude 12° 46' 15", longitude 133° 02' 20") in the Dry season. It should be collected in acid-washed plastic containers as close as is practicable to the start of the test, ie afternoon prior to test commencement, and stored at 4°C overnight. Within 48 hours of collection, the required amount of water for the test should be filtered through a fine pore size filter (eg Whatman No 42, 2.5 μm) capable of removing 'wild' zooplankton. The water should be stored in covered inert containers at 4°C for a maximum period of three weeks to avoid any seasonal variation in water quality and deterioration of water quality in storage (Magela Creek water is poorly buffered and therefore is not stored for long periods). Other natural water types may have different properties and may be held for longer periods – this would be determined for each natural water type.

D.4.2 Filtered Darwin tap water

Darwin tap water is low conductivity, low mineralised good quality soft water. At the ***eriss*** laboratory, water travels from the mains supply predominantly through PVC pipe to two activated carbon filter units. After passing through these the water is then pumped into the roof cavity and is held in two temperature controlled (27 ± 1°C) holding tanks. When water is required for use in culturing or as diluent water for testing, it travels through insulated PVC plastic pipes from the holding tanks to the aquaculture laboratory. General water quality parameters are routinely measured, while water chemistry samples are regularly collected for analysis of major anions and cations.

D.5 Stock solutions

D.5.1 Chemical solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in an acid-washed, polyethylene container and refrigerated (4°C). The source of the stock solution (eg date of preparation, by whom), is marked on the bottle and on an information sheet. Immediately prior to use for test water preparation the stock solution is allowed to equilibrate to room temperature.

D.5.2 Whole effluent solutions

Whole effluent stock solutions are collected from the designated site as close as is practicable to the start of the test, in acid-washed plastic or glass containerers (depending on the chemical properties of the effluent). The sample should be kept sealed, clearly labelled and refrigerated at 4°C until required for test commencement.

D.6 Test solutions

Test solutions are prepared by diluting a stock solution with diluent water. The pH is then adjusted if necessary, using 0.02 M HNO_3 or 0.0125 M NaOH. Test solution concentrations are determined from the results of range-finding studies. Test solutions are prepared in bulk at the start of a test in 2–5 L polyethylene or glass screw-topped containers and refrigerated (4°C) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies significantly when stored for the test period.

D.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the cladocerans are placed, or the cladocerans themselves, should be chemically inert.

D.7.1 Container preparation

All containers (ie bottles, plastic vials etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 μS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least 3 times with Millipore water (< 1 μS cm^{-1});
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with appropriate diluent or control water. Other equipment should be rinsed thoroughly with Milli-Q water (< 1 μS cm^{-1}) before use.

D.7.2 Temperature control

Tests are conducted at 27 ± 1°C using a constant temperature incubator. When removed from the incubator for observation, the temperature of the test containers are maintained at 27 ± 1°C by the use of warming trays on the microscope bench.

D.7.3 Photoperiod control

Tests are conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie 30–100 μmol m^{-2} s^{-1}).

D.7.4 Equipment

- Light-tight constant temperature incubator

- Milli-Q water purification system or equivalent
- A-grade glass volumetric flasks
- Chemicals and reagents
- Analytical balance and weigh boats
- Filter apparatus – 5 L plastic Buchner funnel, 5 L glass flask with side arm, vacuum pump and tubing
- Filter paper – 2.5 µm pore size (Whatman No 42)
- Magnetic stirrer and stirrer bars
- 5 L polyethylene containers (to hold treatment solutions, one per solution)
- Refrigerator for storage of test and stock solutions
- Eighty 45 mL volume polypropylene test vials (eg Nylex type 12) and lids with air holes (for an eight treatment test)
- Eighty 45 mL volume polypropylene test containers and lids with air holes for test solution renewal at 24 h (for an eight treatment test)
- Sixteen 150 mL tall form polypropylene containers (for water parameter measurement for an eight treatment test)
- pH meter, electrical conductivity EC meter and dissolved oxygen meter
- Binocular dissecting microscope with bright field/dark field illumination
- Automatic 0–50 mL dispenser
- Eight clear perspex trays, each capable of holding ten 45 mL test vials (for an eight treatment test)
- Laboratory warming trays, set at 27 ± 1^{o}C, capable of accommodating the clear plastic trays
- Random number generator
- Two plastic trays, one of such a size to hold sixteen 50 mL beakers and the other to hold twenty-four 50 mL beakers
- Pasteur pipettes, with internal tip diameter of ~ 1 mm
- Algae and Fermented Food with Vitamins (FFV) for feeding the water fleas
- Automatic adjustable pipettes (5 µL to 5 mL) and disposable pipetter tips
- Data sheets
- 50–200 mL acid-washed bottles (glass or polyethylene depending on toxicant) with plastic screw caps, for analytical chemistry samples.

D.8 Test environment

The preparation and storage of test solutions, culture of test cladocera, and conduct of tests should be carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with

fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

D.9 Data recording

Test animals are observed and data recorded at 24 h intervals after the commencement of the test (when t = 0 h). Observations made at the end of the first 24 h period are designated as Day 1 observations; at the end of the second 24 h period, Day 2 observations etc. Water quality parameters are measured and adjusted (where appropriate) and recorded at the beginning and end of each 24 h period, and are designated as Fresh Water Day 0, 24 h-old Water Day 1, respectively, and so forth during the test.

D.10 Test procedure

Day 0 (ie starting day of the test t = 0 h)

1. Prepare the test solutions (as outlined in Section D.6) and equilibrate to 27 ± 1°C.
2. Prior to the expected commencement time of the test, isolate 24 mature female cladocerans with eyed young visible in their brood chamber (or with their second brood if neonates less than 6 hours old are already present) randomly into three glass crystallising dishes containing diluent water. Place the crystallising dishes on the warming tray to maintain at test temperature. The test should commence within six hours of the birth of brood two neonates.
3. To each test vial add 30 µL FFV (Fermented Food with Vitamins) and the required amount of algae (6×10^6 cells; see Appendix D.6). Then immediately dispense 30 mL aliquots of each test concentration (normally 8) into 10 appropriately labelled replicate 45 mL vials (ie 10 × 30 mL for each concentration) and allow to equilibrate to incubator temperature (27 ± 1°C). Arrange vials in the eight concentration groups on the clear Perspex vial trays. Also dispense and cap a sample of each test solution (50–100 mL with food and algae added at the correct ratio) into appropriately labelled 150 mL containers for the measurement of 'fresh' water quality parameters (pH, conductivity and dissolved oxygen).
4. Using a microscope and Pasteur pipette with tip < 1mm, pick out one test neonate from the isolated stock, and drop gently into control replicate 1 without touching the test solution.
5. Repeat for the first vial of each test concentration, working up in concentration and ending with the highest concentration.
6. Discard the used pipette and obtain a clean one if the test solution was touched at any point.
7. Repeat steps 4–6 for the other vials until all ten vials for each concentration contain 1 neonate.

   Note: more than one person can distribute test neonates simultaneously
8. Under the microscope cross-check each vial to ensure that there is one neonate in each vial.
9. When one neonate has been distributed into each of the 80 vials, cover vials with plastic caps with air holes and place them in the random order for that day (see Section D.11).

10. Place the eight trays in the incubator in their random order for that day (see Section D.11).
11. Completion of this stage constitutes the start of the test (time = 0 hours) and is recorded on the information sheet in the test folder.
12. Measure water quality parameters ie pH, conductivity and dissolved oxygen.

Note: Whenever test dishes are removed from the incubator maintain them at 27^{o}C (eg by placing them on a warming tray).

Day 1

13. Dispense test solutions into appropriately labelled vials (10 × 30 mL of each test concentration and the FFV and algae food supplements. Also dispense a 50−100 mL sample of each solution and food supplements for pH, conductivity and dissolved oxygen measurement, as in step 3). Cover vials and allow dispensed solution to equilibrate to 27 ± 1^{o}C in an incubator.
14. 24 hours after the commencement of the test, remove trays from incubator and sort the test vials into numerical order for each concentration group.
15. For each concentration group, observe and transfer each live test flea into the appropriate vial of fresh water, cover with caps with air holes immediately and:
    a) Record whether the test flea is alive, dead or missing;
    b) Record whether eyed-young are seen in the brood chamber;
    c) Note if there are any neonates present, and if so how many are dead;
    d) Make any other observations which suggest that the water fleas are not developing normally eg haemorrhaging, small size etc.
16. Observations are recorded as Day 1 observations.
17. To avoid observer bias, each day select a different concentration group to observe first.
18. Keep the old water aside to count neonates later (usually present from Day 2).
19. Obtain a clean pipette if observing a lower concentration solution than the previous one.
20. When test water fleas from all concentration groups have been transferred into fresh concentrations, place vials into the random order for that day, and place trays into the incubator in the random order for that day (see Section D.11).
21. For each vial containing the previous day's test solution (ie the 'old' vials,), 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, the live neonates will swim. Pipette up and remove each neonate as it is counted.
22. After the neonate count is complete, a sample of the old test solution for each concentration is collected, as the '24-hour-old water' for water parameter measurement.
23. Measure the physical water quality parameters (ie pH, conductivity, dissolved oxygen) of the '24-hour-old' (Day 1) and 'fresh' (Day 1) test solution samples, and record for the appropriate day

Day 2–5

24. Repeat steps 13–23 (ie at 24 hour 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).

Note: On each day a new set of random numbers must be used for the position of each vial and tray position in the incubator for the next 24 h period (Section D.11).

Day 5–6

25. Count and record observations on test water fleas and the number of neonates produced (total and number dead), but do not dispense or tranfer test water fleas to fresh test solution.

26. Measure the water quality parameters for the 24-hour-old water and record as Day 5 or 6 (depending on which day the test finishes).

Test is complete when three broods have been produced by 80% or greater of control water fleas (usually Day 5 or 6 – if not, continue test into Day 7 and check results) and have been counted.

D.11 Randomisation

On each day a new set of random numbers must be used to assign the position of each vial on each plastic tray, and the position of each plastic tray in the incubator. Randomness is an important component of the experimental design. Random distribution of cladocerans is achieved via steps 4–10. The vials are randomly assigned to positions on trays each day and they will also have a random position in the incubator. Random numbers are obtained from a random number table or generator for each day of the test; a set of random numbers is unique for each test and is not to be reused. When the water fleas have to be observed, the vials and trays can be sorted into numerical order for greater convenience. This avoids the continual changing of glass pipettes by working through the water changes from a lower to a higher chemical concentration. At the end of the water changes the Petri dishes are again randomly placed on trays and returned to the incubator.

D.12 Avoiding bias

To avoid operator bias there should be at least two people present to carry out the test when possible. The two operators alternate the concentrations they observe each day. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section D.13, should be used.

D.13 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Uranium (added as uranyl sulphate) is used in a concentration range from 0.5–50 ug/L. Natural Magela Creek water is used as diluent. The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to uranium. If the value falls

outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

D.14 Acceptability of test data

The data set of the test (ie all data from each group) is considered acceptable if:

1. The recorded temperature of the incubator remains within the prescribed limits;
2. 80% or more of the test control cladocera are alive, female and have produced three broods at the end of the test period;
3. The result of reference toxicity testing is within the set limits.

The data of each test group is considered acceptable if:

1. Reproduction in the control averages 30 or more neonates surviving per female over the test period;
2. The recorded pH is within the prescribed limits (usually ± 0.2 unit of Day 0 values for each test concentration);
3. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 27°C; and
4. The conductivity for each test solution was within 10% of the values obtained on Day 0;
5. No more than 20% of parental cladocerans are reported missing in any treatment (except if all other cladocerans are dead in that group).

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

D.15 Analysis of test data

Statistical endpoints for most tests are determined using the data analysis package, ToxCalc™ Version 5.0.23D (Tidepool Software). This package enables all required toxicity indices to be calculated using parametric and non-parametric statistical methods. Analysis of data is performed using a range of methods including Steels Many-One Rank Test (non-parametric) and Dunnett's Multiple Comparison Test (parametric). LOEC and NOEC values are obtained using hypothesis testing ($p < 0.05$) and point estimates are calculated using Linear Interpolation for Reproduction tests. Point estimates are compared using Standard Error of Difference (Sprague & Fogels 1976). Where a toxicant is not used (eg comparing diluent or culture water types), data are analysed in Minitab, using one and two-way Analysis of Variance (ANOVA) to calculate significant differences ($p < 0.05$) and *post-hoc* analysis using Tukeys Pairwise Analysis to locate the significant differences. Origin can also be used to calculate point estimates, ie EC/IC/LC_{50}. Prior to use in Origin, data are manipulated in Microsoft Excel to derive averages, and 95% confidence intervals.

Appendix D

BTT-D: Cladoceran reproduction test – Stock culture maintenance

A.D.1 Primary cladoceran stock culture

Water fleas are kept individually in small vials (45 mL plastic vials with snap-on lids, lids have 2 air-holes) and are transferred to 30 mL aliquots of fresh water daily. The water fleas are kept in filtered Magela Creek water and filtered Darwin tap water, fed FFV (Fermented Food with Vitamins) and algae and are kept in the incubators. 24-36 vials are kept at all times, but further vials may need to be maintained depending on experimental demands. Presently, water flea cultures are kept in both Magela Creek water and filtered Darwin tap water (following the relocation of the ecotoxicology laboratory from Jabiru to Darwin). A period of 12 months acclimation of cultures into filtered Darwin tap water has continued to give variable results on survival and reproduction. Therefore, at the time of writing this report it was decided to include both culturing water types.

Every 3-4 days, neonates are collected to restart the stock (preferably from neonates produced in the second brood), to ensure continuation of species line. As for toxicity testing, neonates from the first brood are never used to restart the stock – the second brood is used preferentially over the third due to increased number of males present in the third brood (C Camilleri, pers comm).

A.D.2 Secondary cladoceran stock culture

A secondary stock of cladocera are maintained in filtered Magela Creek water and filtered Darwin tap water (refer to A.D.1). Clear glass food grade bowls are filled with 1–2 L of culture water and kept in a separate location, as a precaution against contamination or accidents. This is a monospecific culture of cladocerans. Detailed notes are recorded in laboratory log books.

The secondary cladocera culture are fed every second day with algae and FFV (approximately 450 μL). When the second brood is produced by the primary cladocera stock culture, the excess water fleas are used to restart the secondary culture. The original water fleas are removed, the bowl cleaned and water renewed. The primary cladocera (and water) are added, along with approximately 50 cladocera from the secondary culture. Detailed notes are recorded in laboratory log books.

A.D.3 Sexual reproduction in cladoceran cultures

If the animals are stressed, which occurs if the culturing conditions are below optimum (eg polluted water, little food, frozen algae fed to primary cultures etc) the adult diploid female may produce both diploid *male* and diploid *female* young.

In sexual reproduction mature diploid males mate with mature diploid females, and the females produce a different sort of egg, a resting egg. These special eggs are protected by thickenings of the brood chamber, which is called an ephippium. The ephippium and its eggs lie dormant (even after the adult female dies) until favourable conditions occur, allowing the eggs to hatch. The eggs have genes from both sexes, ie heterozygous, thus enabling the cladocera to better adapt to its changing environment, and ensure survival of the species. This

phenomenon (the occurrence of a seasonal variation in the external appearance of specimens) is known as cyclomorphosis, and is a characteristic of many species of Cladocera including *M. macleayi*.

It has not been determined if there is a difference in survival/behaviour etc between male and female cladocera in test conditions. However, based on the above information, it is assumed that males may show a greater tolerance to adverse conditions, and hence may not react representatively to toxicants. In addition, as this is a reproduction test, only females can be used. This laboratory has not used males for testing purposes.

A.D.4 Recommended husbandry of cladocera

An incubator set to 27 ± 1°C houses a stock of asexually-reproducing (parthenogentic) water fleas kept in separate vials (usually 24–36 animals). These vials are 45 mL screw-capped vials with two 2 mm diameter ventilation holes per cap (Nylex type 12, Selby Scientific and Laboratory Equipment, are suitable). Four days prior to the test (eg on Thursday if the test starts on the following Monday) neonates from the second brood are collected for use as parents of the test neonates. The water and food is changed daily for each vial, and neat accurate records maintained on the culture health. Water is changed by transferring the water fleas (adults only if any brood present) using a pipette into clean vials. Each vial has 30 mL of fresh diluent water, with the water equilibriated to 27 ± 1°C prior to transfer of the water flea. Fermented pellet food is added as a suspension at a rate of 30 µL/30 mL/day, and algal cells at a rate of 6×10^6 cells/30 mL/day. Neonates from the first brood are discarded. Neonates from the second brood are collected for the test animals (brood three neonates are avoided due to increased presence of males).

A.D.5 Fermented Food with Vitamins (FFV)

Five grams of a commercially-available proprietary pelleted cichlid food (Wardley Products, New Jersey) and 0.25 g of dried and powdered alfalfa are homogenised in 250 mL of Milli-Q water for five minutes using a domestic food-blender. The mixture is then bubble-aerated for 2–3 days at ambient water temperature (25–34°C), after being covered with a fine mesh to ensure it does not become contaminated by flies, dust etc, and to allow gas exchange. At the end of this fermenting period (the mixture is determined to be pungent rather than putrid to smell), the mixture is collected into a beaker and placed at 4°C for 1 hour and allowed to settle. The supernatant (approximately 150 mL) is then decanted carefully into another clean beaker and 0.3 g of calcium pantothenate and 1.5 mL of a stock Vitamin B_{12} solution (100 mg/L) is added to this beaker (Murphy 1970, Keating 1985). This is then divided into aliquots of about 1 mL in plastic vials, capped and frozen and clearly labelled with preparation date. An aliquot is thawed when needed, and resuspended by gentle shaking before use. The aliquot can then be refrigerated (4°C) but must be discarded and replaced with a fresh aliquot one week after thawing. Records of FFV replacement are kept in the water flea culture log book to ensure different operators can determine the age of the FFV and when to discard old batches.

A.D.6 Protocol for mass culture of algae

Two types of algal cultures are maintained:

(i) stock or ‘starter’ cultures

(ii) mass or ‘food’ cultures

The alga was isolated from Georgetown Billabong, and has been tentatively identified as a *Chlorella* sp. described by Ling (pers comm), and cultured in the laboratory.

All steps outlined below should be carried out aseptically in a laminar flow cabinet using sterile media and glassware. Starter cultures are placed in the growth cabinet (approximately 40–50 µmol m^{-2} s^{-1}) set on a 12:12 light/dark cycle at 29 ± 1°C and are kept on a constant gentle-motion agitator plate. Culture flasks for mass culture of algae for food are placed in the growth cabinet (40–50 µmol m^{-2} s^{-1}), set on a 12:12 light/dark cycle at 27 ± 2°C and are continuously gently swirled. A mass culture should be prepared at least monthly.

A.D.6.1 Method for the preparation of algae using MBL

1. Approximately 5–6 days before commencing a mass culture, transfer ~ 2 mL of algae from a culture five to seven days old into a 250 mL Erlenmeyer flask to which 100 mL of MBL media has been added. Cells in this new starter culture should be exponentially growing by the time they are used to inoculate the harvest food culture.
2. To prepare media for the algae culture, make up 4–6 L of MBL medium (see table A.D.1) and dispense 1 L aliquots in 2 L Erlenmeyer flasks and adjust pH to between 7.1 to 7.3 using HCl acid. Stopper the flask with non-absorbent cotton wool bung and cover with aluminium foil to prevent contamination by airborne particles. Autoclave for 20 minutes (see below for details on MBL preparation). Allow the medium to sit at room temperature for at least 1 day before use to ensure adequate gas exchange.
3. Add 10 mL of starter culture to each 2 L flask of harvest media using a sterile pipette in the Laminar Flow Cabinet to inoculate the harvest culture. Place the flask in the growth cabinet, culture for 7–10 days (until harvest appears quite green) and then harvest the algae.

Table A.D.1 MBL medium solutions and procedure

	Ingredient	Stock Solution	Media Solution
1	Tris Buffer	100 g/L	5 mL/L
2	$NaNO_3$	85.24 g/L	1 mL/L
3	$CaCl_2.2H_2O$	36.76 g/L	1 mL/L
4	$MgSO_4.7\ H_2O$	36.97 g/L	1 mL/L
5	$NaHCO_3$	12.6 g/L	1 mL/L
6	K_2HPO_4	8.72 g/L	1 mL/L
7	Na_2EDTA	4.36 g/L	1 mL/L
8	$FeCl_3.6H_2O$	0.727 g/L	1 mL/L
9	Vitamins	*See below*	1 mL/L
	Cyanocobalamin (Vitamin B12)		
	Thiamine hydrochloride (Vitamin B1)		
	d-Biotin (Vitamin H)		
10	**Trace metals**	In 1 L add:	1 mL/L
	$CoCl_2.6H_2O$	10 mg/L	
	$CuSO_4.5H_2O$	9 mg/L	
	$Na_2SiO_3.5H_2O$	7 mg/L	
	$MnCl_2.4H_2O$	180 mg/L	
	$ZnSO_4.7H_2O$	22 mg/L	

Vitamin Stock

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

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

Note: These stocks can be stored frozen.

Preparation of Stock:

1. Weigh 0.050g thiamine into a dry 250 mL volumetric flask.
2. Add a few mL of Milli-Q water to dissolve.
3. Add 2.5 mL of the above biotin stock and 2.5 mL of the Vitamin B12 stock.
4. Make up to 250 mL with Milli-Q water. Use 1 mL of this stock per litre MBL media.

AD.6.2 Procedure for making media

1. Add 1 ml of each stock solution per litre of Milli-Q water, except Tris Buffer – add 5 mL/L.

Note: These ingredients are stored at 4°C, and require replacing at 18–24 month intervals.

2. Adjust media to pH 7.1–7.3 using 25% HCl.
3. Pour MBL media into 4–6 x 2 L flasks, such that there is 1 L per flask for the harvest culture.
4. Use a cotton bung to plug the top of the each flask. Cover the bung and mouth of the flask with aluminium foil. Record the date the media is autoclaved and media type on a strip of autoclave tape and place on alfoil.
5. Autoclave at 121^{o}C for 20 min.
6. Allow the media to cool to room temperature before inoculating.
7. Media may be stored at room temperature while not in use.

AD.6.3 Protocol for harvesting algal cells

1. Aseptic techniques are not practical in the harvesting of algal cells but the work area should be as clean as possible. All dilution water (eg Magela Creek water) used in the following steps is sterilised by autoclaving.
2. 4 x 500 mL plastic tubes are filled with 400–500 mL of the algae culture. The tubes are placed in a refrigerated centrifuge fitted with a swing-out head. Tubes placed opposite each other are pair-wise balanced or matched to the nearest 0.1 g, both for this step and all other steps. Any remaining algae culture can be spun in used tubes after the supernatant has been removed and the resuspended pellet transferred. The centrifuge is set to a rotor speed of 3500 rpm and a temperature of 15°C. A spinning time of 20 minutes is adequate.
3. The supernatant is removed through suction, using a glass Pasteur pipette directly attached to a venturi suction via flexible plastic tubing. Use of the venturi suction allows controlled removal of any bacteria growth that may become evident as a layer on the surface of the algal pellet. A sufficient quantity of water (≈5 mL) is left behind to allow resuspension of the pellet.
4. The resuspended pellets are combined and transferred into the minimum number of tubes possible – 60 mL tubes are used after the volume is significantly reduced. The tubes are spun at 2800 rpm for 20 minutes at 15°C.

5. The empty tubes are rinsed by adding 20–30 mL of sterile dilution water, capped and shaken to ensure total resuspension of pellet. To keep the volume of rinsing water used to a minimum, the same aliquot is transfered from one tube to another, and eventually to a final tube to be re-spun. This rinsing procedure is repeated three times, or until the final aliquot appears clear.
6. The above procedure is repeated until all algal cells are concentrated into one tube. These cells are then washed three times with sterile dilution water to remove any trace metals (from the culture media) by spinning, removing the supernatant, adding approximately 50 mL sterile dilution water and shaking sufficiently to resuspend the pellet.
7. The final rinsed pellet from step 6 is finally resuspended in approximately 50 mL of sterile dilution water.
8. The algal density of the suspension must be measured to calculate the required volume needed to be added to *Moinodaphnia macleayi* treatment vials to attain the pre-determined known cell density of 2×10^5 algae/mL. To measure algal density (see also Padovan 1992), transfer 1 mL of the suspension to a 100 mL volumetric flask and make up to the mark using sterile dilution water. Seal with Parafilm and mix well by inverting five times. Quickly break the seal and load the haemocytometer for a cell count. If the algal density is too high, try a greater dilution (eg add 0.5 mL concentrate to a 250 mL volumetric flask). Count at least 100 cells in each of four chambers (re-seal and invert five times before loading the haemocytometer again), average the values and back–calculate to determine the algal density of the concentrate.
9. The algal suspension should be divided into 2 mL aliquots and stored in the dark at 4°C. The algal feeding aliquot of 30×10^6 cells should always have a volume range of 20-500 µL. If not, the algal concentrate should be re-centrifuged and the volume adjusted.

PROTOCOL SHEET

Project Name Djalkmara pre-release testing **Project Number:**

Test Number: 611D **Test Name:** Clad_Djalk_02 **Start Date:** 17/12/02

BTT: D-Cladoceran reproduction test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	3000 mL	0 mL
B	0.3%		9 mL
C	1.0%		30 mL
D	3.2%		96 mL
E	10%		300 mL
F	32%		960 mL
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: 611D **Test Name:** Clad_Djalk_02

Toxicant: Djalkmara Billabong **Diluent:** Magela creekwater

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	As per lab manual	As per lab manual
Site	Djalkmara Billabong pump station	Georgetown
Transportation	22 L jerry cans	22 L jerry cans
Comments	Collected by XX	Collected by XX

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: Chemical weighed out by:

Balance used: Stock solution prepared by:

TEST DETAILS			
BTT	D		
Species:	*Moinodaphnia macleayi*		
Start Date:	17/12/02		
Start Time:	10:00am		
Started by:	XX		
Incubator no:	Beige-1		
Temps OK?	yes		
Test waters prepared by:	XX		
Supervisor:			
Chem. analysis: **J/N,S/N & date** **Submitted by:**	NTEL – metals AGAL – TOC, alkalinity XX		
Departure from BTT / comments:			

FRESH water parameters

Test No: 611D	**Test:** Clad_Djalk_02	**Species:** *Moinodaphnia macleayi*	
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong	
PH:	**Cond:**	**pH:**	**Cond:**

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**					**Observer / Date:**
0	pH											
	Cond											
	DO											
1	pH											
	Cond											
	DO											
2	pH											
	Cond											
	DO											
3	pH											
	Cond											
	DO											
4	pH											
	Cond											
	DO											
5	pH											
	Cond											
	DO											

QC: pH stays w/I ±0.2 unit of Day 0 values for each Conc.; Cond for each test soln is w/I 10% of Day 0 values; DO conc. >70% air saturation value for each conc.

24 hour OLD water parameters

Test No: 611D	**Test:** Clad_Djalk_02	**Species:** *Moinodaphnia macleayi*
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**			**Observer/ Date:**
1	pH									
	Cond									
	DO									
2	pH									
	Cond									
	DO									
3	pH									
	Cond									
	DO									
4	pH									
	Cond									
	DO									
5	pH									
	Cond									
	DO									
6	pH									
	Cond									
	DO									

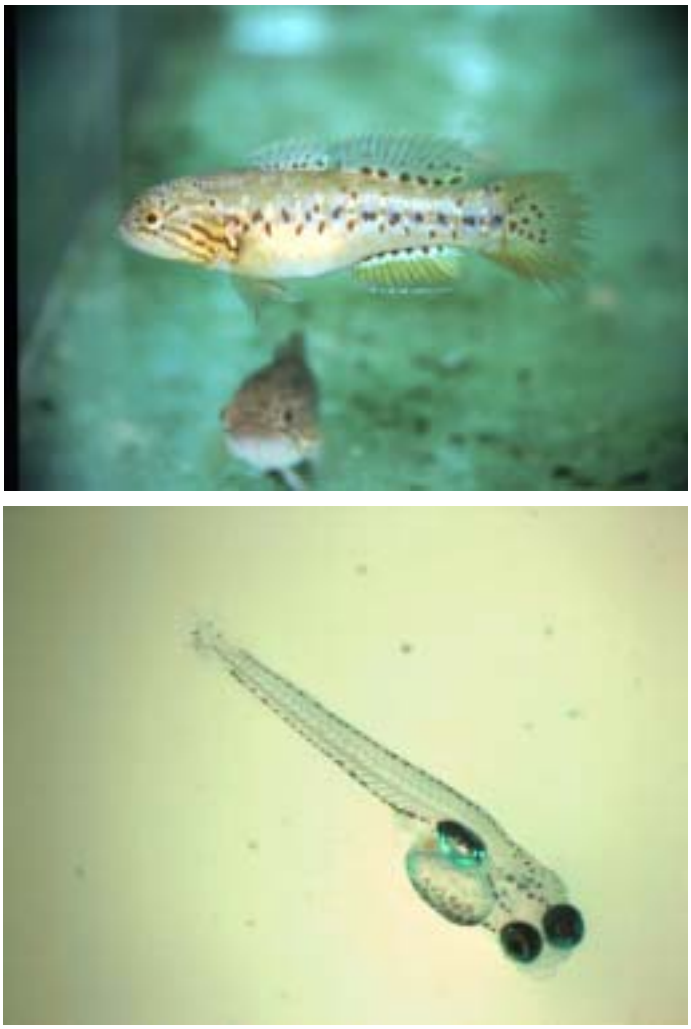
FLEA REPRODUCTION TEST

Test No.: 611D **Species:** *Moinodaphnia macleayi* **Concentration:** CONTROL

Test Name: Clad_Djalk_ 02 **Start Date:** 17/12/02

Replicate/Observation		1	2	3	4	5	6	7	8	9	10	
Day 1	**Alive/Dead (A/D):**											**Observer/Date**
	Brood size:											
	Juvenile mortality											**Checked by**
	Comments:											
Day 2	**Alive/dead (A/D):**											**Observer/Date**
	Brood size:											
	Juvenile mortality											**Checked by**
	Comments:											
Day 3	**Alive/Dead (A/D):**											**Observer/Date**
	Brood size											
	Juvenile mortality											**Checked by**
	Comments:											
Day 4	**Alive/Dead (A/D):**											**Observer/Date**
	Brood size											
	Juvenile mortality											**Checked by**
	Comments:											
Day 5	**Alive/Dead (A/D):**											**Observer/Date**
	Brood size											
	Juvenile mortality											**Checked by**
	Comments:											
Day 6	**Alive/Dead (A/D):**											**Observer/Date**
	Brood size											
	Juvenile mortality											**Checked by**
	Comments:											
Sum of broods:												

BTT-E: Purple-spotted gudgeon sac-fry survival test



Mogurnda mogurnda Richardson, adult fish at top, newly hatched larvae below

BTT-E: Purple-spotted gudgeon sac-fry survival test

E.1 Objective

The objective of a test series (ie 3–4 definitive tests) is to determine the concentrations of a specified chemical or whole effluent that shows:

d) The No Observed Effect Concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the 10% Bounded Effect Concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk 1993);

e) The Lowest Observed Effect Concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response becomes significantly ($P \leq 0.05$) lower than that of the control (Ahsanullah & Williams 1991);

f) The Median Lethal Concentration, which is defined as a 50% decline in the test parameter (LC_{50}). For this test it is measured as the LC_{50} on the survival of *M. mogurnda* (Purple-spotted gudgeon) sac-fry over 96 h.

E.2 Principle of the test

Newly hatched sac-fry (<10 h old) are exposed to a range of chemical concentrations for 96 h. Observations of any sac-fry mortality are recorded at 24 h intervals. The method is based on the 'Gudgeon embryo larval test' described by Hyne et al (1996).

E.3 Test organism

The test species is *Mogurnda mogurnda* Richardson (Teleostomi, Eleotrididae) commonly known as the Purple-spotted gudgeon or Northern trout gudgeon (Merrick & Schmida 1984). This carnivorous species is widely distributed throughout northern Australia (Merrick & Schmida 1984). Laboratory stocks are regularly collected from wild populations in Magela Creek upstream of any mining activities. The recommended husbandry method for *M. mogurnda* is described in Appendix E.1. Sac-fry < 10 h old are used to commence the test. Neither the embryos nor sac-fry are treated for fungus, but should be free from overt disease or gross morphological deformity. No feeding is required during the test, as the animals obtain sufficient nutrition from the attached yolk-sac. Original test gudgeons were collected from Magela Creek, with recent cultures restocked from Radon Springs in September 2002.

E.4 Dilution water

There are two diluent water types which are routinely used. In addition, dechlorinated filtered Darwin tap water can be used as an analogue of low conductivity, low mineralised soft water. The aim of the test will determine which water type is used.

E.4.1 Synthetic water

'Synthetic' water simulates the inorganic composition of Magela Creek water during the Wet season. Magela Creek water is very soft, slightly acidic and has a low buffering and complexation capacity. These qualities make synthetic softwater particularly useful in providing a worst case toxic scenario, as under these conditions many toxicants (particularly metals) are highly available to the test organism. The ionic composition of Magela Creek water is representative of sandy braided streams throughout much of the Wet/Dry tropics. The synthetic water is prepared by adding analytical grade reagents to Milli-Q water (< 1 µS cm^{-1}) (table E.1) in acid-washed polyethylene containers, as close as practical to the start of the test (see appendix A.E.6). The pH of the test water is adjusted to the required level (in this case 6.0 ± 0.15 at 27 ± 1°C) with 0.02 M HNO_3 or 0.0125 M NaOH. The test water is stored in sealed polyethylene containers and refrigerated (4°C) until use.

Table E.1 Mean nominal composition of the synthetic water

Physico-chemical parameter	Total concentration
pH	6.0 ± 0.15[a]
Temperature (°C)	27 ± 1[a]
Na	1.00 mg L^{-1}
K	0.37 mg L^{-1}
Ca	0.45 mg L^{-1}
Mg	0.60 mg L^{-1}
Cl	2.32 mg L^{-1}
SO4	3.12 mg L^{-1}
HCO_3	2.63 mg L^{-1}
NO_3	0.07 µg L^{-1}
Fe	100 µg L^{-1}
Al	70 µg L^{-1}
Mn	9.7 µg L^{-1}
U	0.10 µg L^{-1}
Cu	0.70 µg L^{-1}
Zn	0.70 µg L^{-1}
Pb	0.12 µg L^{-1}

a pH and temperature were tightly regulated as described in text

E.4.2 Natural stream water

Natural stream water is the receiving water taken upstream of the waste water discharge outlet at Magela Creek upstream of Georgetown pumping station (latitude 12° 40' 28", longitude 132° 55' 52") – Wet season *or* Bowerbird Billabong (latitude 12° 46' 15", longitude 133° 02' 20") – Dry season. It should be collected in acid-washed plastic containers as close as is practicable to the start of the test, ie afternoon prior to test commencement, and stored at 4°C overnight. Within 48 hours of collection, the required amount of water for the test should be filtered through a fine pore size filter (eg Whatman No 42, 2.5 µm) capable of removing 'wild' zooplankton. The water should be stored in covered polyethylene containers at 4°C for a maximum period of three weeks.

E.4.3 Filtered Darwin tap water

Darwin tap water is low conductivity, low mineralised good quality soft water. At the ***eriss*** laboratory water travels from the mains supply predominantly through PVC pipe to two activated carbon filter units. After passing through these the water is then pumped into the roof cavity and is held in two temperature controlled (27 ± 1°C) holding tanks. When water is required for use in culturing or as diluent water for testing, it travels through insulated PVC plastic pipes from the holding tanks to the aquaculture laboratory. General water quality parameters are routinely measured, while water chemistry samples are regularly collected for analysis of major anions and cations.

E.5 Stock solutions

E.5.1 Chemical solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in an acid-washed, polyethylene container and refrigerated (4^{o}C). The source of the stock solution (eg date of preparation, by whom), is marked on the bottle and on a test information sheet. Immediately prior to use for test water preparation the stock solution is allowed to equilibrate to room temperature.

E.5.2 Whole effluent solutions

Whole effluent stock solutions are collected from the designated site as close as is practicable to the start of the test, in an acid-washed plastic or glass container (depending on the chemical properties of the effluent). The sample should be kept sealed, clearly labelled and refrigerated at 4°C until required for test commencement.

E.6 Test solutions

Test solutions are prepared by diluting a stock solution with diluent water. The pH is then adjusted if necessary, using 0.02 M HNO_3 or 0.0125 M NaOH. Test solution concentrations are determined from the results of range-finding studies. Test solutions are prepared in bulk at the start of a test in 2–5 L polyethylene or glass screw-topped containers and refrigerated (4^{o}C) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies significantly when stored for the test period.

E.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the sac-fry are placed, or the sac-fry themselves, should be chemically inert.

E.7.1 Container preparation

All containers (ie bottles, Petri dishes and lids etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 μS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least 3 times with Millipore water (< 1 µS cm^{-1});
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with appropriate diluent or control water. Other equipment should be rinsed thoroughly with Milli-Q water (< 1 µS cm^{-1}) before use.

E.7.2 Temperature control

Tests are conducted at 27 ± 1°C using a constant temperature incubator. When removed from the incubator for observation, the temperature of the test containers are maintained at 27 ± 1°C by the use of warming trays on the microscope bench.

E.7.3 Photoperiod control

Tests are conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie. 30–100 µmol m^{-2} s^{-1}).

E.7.4 Equipment

- Light-tight constant temperature incubator
- Milli-Q water purification system or equivalent
- A-grade glass volumetric flasks
- Chemicals and reagents
- Analytical balance and weigh boats
- Calibrated mercury thermometer
- Filter apparatus – 5 L plastic Buchner funnel, 5 L glass flask with side arm, vacuum pump and tubing
- Filter paper – 2.5 µm pore size (Whatman No 42)
- Magnetic stirrer and stirrer bars
- 5 L polyethylene containers (to hold treatment solutions, one per solution)
- Refrigerator for storage of test and stock solutions
- 90 mm diameter disposable plastic Petri dishes with lids (3 replicates per solution)
- 20 x 90 mm diameter disposable plastic Petri dishes without lids for cleaning
- 150 mL tall-form polypropylene containers (for water parameter measurement – one per solution)
- 45 mL plastic vials (eg Nylex type 12), for aliquoting 30 mL to respective treatment Petri dishes, with 3 replicates per test concentration

- pH meter, electrical conductivity EC meter and dissolved oxygen meter
- Binocular dissecting microscope with bright field/dark field illumination
- Automatic 0–50 mL dispenser
- Three clear perspex trays, each capable of holding Petri dishes, with position numbers 1 to 24 marked (extra trays can be added if more test solutions are run in a test)
- Laboratory warming trays, set at 27^{o}C, capable of accommodating the clear plastic trays
- Random number generator
- Two plastic trays, one of such a size to hold sixteen 45 mL beakers and the other to hold twenty-four 45 mL beakers
- Wide mouth Pasteur pipettes, with internal tip diameter of > 2 mm
- Data sheets
- 50–200 mL acid-washed bottles (glass or polyethylene depending on toxicant) with plastic screw caps, for analytical chemistry samples
- MS222 euthanasing solution

E.8 Test environment

The preparation and storage of test solutions, culturing of gudgeons, and conduct of tests should be carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

E.9 Data recording

Test animals are observed and data recorded at 24 h intervals after the commencement of the test (when t = 0 h). Observations made at the end of the first 24 h period are designated as Day 0 observations; at the end of the second 24 h period, Day 1 observations etc. Water quality parameters are measured and adjusted (where appropriate) and recorded at the beginning and end of each 24 h period, and are designated as Fresh Water Day 0, 24 h old Water Day 1, respectively, and so forth during the test.

E.10 Test procedure

Day 0 (ie start day of test)

1. Prepare the test solutions (as outlined in Section E.6) and allow to equilibrate to 27 ± 1^{o}C.
2. Isolate approximately sufficient suitable test sac-fry into three Petri dishes (one dish for each replicate) filled with diluent water and equilibrate to 27 ± 1^{o}C (eg a six concentration test requires: 10 sac-fry per dish x 6 concentrations x 3 replicates = 180 sac-fry + ~ 30 extras). At the commencement of the test, the fry must be < 10 hours old from hatching. The sac-fry is seen as a developed, hatched fry lying on the bottom of the hatching container, with a prominent yolk-sac and black-eye pigmentation visible.
3. Dispense 30 mL aliquots of each test concentration (normally eight) into three appropriately labelled replicate Petri dishes (ie 3 x 30 mL for each test solution), and

arrange in three replicate groups on clear plastic trays (eg. Control replicate 1 to X µg L^{-1} on Tray 1).

4. Dispense 60 mL of each test concentration into the appropriately labelled (ie 'New water A' etc) 150 mL polypropylene containers for water parameter analysis.
5. Using a microscope and wide mouth Pasteur pipette, pick out one sac-fry from the isolated stock and place into control replicate 1, without allowing the pipette tip to contact the test concentration.
6. Repeat for remaining test concentrations of replicate 1, working up in concentration, and ending with the highest concentration.
7. Discard the used pipette and select a new one if at any time it comes in contact with any of the test concentrations.
8. Repeat steps 5–7 until all test dishes for that replicate group contain ten sac-fry.
9. Observe each dish under the microscope to ensure that there are ten sac-fry in each dish, and replace any sac-fry that are damaged in any way (eg disrupted yolk sac, haemorrhaging etc).
10. Repeat steps 5–9 for the remaining two replicate groups.

Note: More than one person can distribute test sac-fry simultaneously, with the distribution appropriately split into replicate groups.

11. Cover the dishes and place them in the random order for that day (Section E.11) eg in positions 1 to 24 on the perspex trays.
12. Place trays in the incubator.

Completion of this stage constitutes the start of the test (time = 0 h).

Note: Whenever test dishes are removed from the incubator maintain them at 27°C (eg by placing them on a warming tray).

13. Measure water quality parameters ie pH, conductivity and dissolved oxygen.

Note: To avoid observer bias, select a different replicate to observe each day. Also, commence observations with the next highest chemical concentration to that observed on the previous day (Section E.12).

Day 1

14. Dispense 70 mL of test solutions into appropriately labelled 150 mL vials and check the pH, conductivity and dissolved oxygen. If they are not within the prescribed limits, adjust accordingly using 0.02 M HNO_3 (625 µL per 500 mL) or 0.0125 M NaOH (1 g per 500 mL and diluted by 1/4) if necessary.
15. When the pH range is established, dispense 35 mL aliquots of test solution into appropriately labelled 45 mL vials (3 x 35 mL of each solution). Cover dispensed solutions and allow them to equilibrate to 27°C.
16. Twenty-four hours after the commencement of the test, remove the trays from the incubator, arrange the test dishes into replicate groups, observe under the microscope and record as Day 1 observations:
    a) The number of live sac-fry;
    b) The number of dead and/or sac-fry with visible fungus contamination; and

c) Any other observations that suggest that the sac-fry are not developing normally eg. spine curvature etc.

Note: To avoid observer bias, each day select a different replicate group to observe first

17. After observing a dish, the test solution is renewed as follows:

    a) Most of the solution in the test dish is carefully emptied into a second Petri dish (or cleaning dish) with a gentle swirling action, tilting the dish to one side to pool the sac-fry in a small area;

    b) Enough of the appropriate fresh test solution (5 mL) is immediately added to cover the bottom of the test dish, the swirling process is repeated, and the solution pipetted or carefully tipped into the cleaning dish. The sac-fry are kept submerged at all times by tilting the dish;

    c) The remaining fresh solution (30 mL) is then immediately added to the test dish;

    d) Any live sac-fry that are transferred to the cleaning dish at this stage are carefully put back into the test dish using a wide mouth pipette;

    e) Any dead sac-fry in the test dish are removed with a pipette before renewal of test solution, with care taken to minimise removal of test solution. A fresh pipette is obtained after the removal of dead sac-fry;

    f) The cleaning dish is checked again for sac-fry, with any found being returned to the test dish; and

    g) The solution in the cleaning dish is collected for measurement of the physical water quality parameters in each treatment after 24 h (ie 'Old water Day 1).

    Note: To ensure that cross-contamination does not occur, obtain a new pipette and cleaning dish whenever a dish of lower chemical concentration is cleaned after a high concentration.

18. After all dishes have been observed and test solutions renewed, place dishes in the random order for that day (see Section E 11), and return trays to the incubator.

19. Measure the physical water quality parameters (ie pH, conductivity, dissolved oxygen) at the end of Day 1.

Day 2–3

20. Repeat steps 14–18 (ie 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).

21. Measure the physical water quality parameters of the 24 h water and record for the appropriate day.

Note: On each day a new set of random numbers must be used for the position of each Petri dish in the incubator for the next 24 h period (Section E.11).

Day 4 (ie at 96 h)

22. Count and record observations for each test dish 96 h (4 x 24 h) after the start of the test. Do not renew test solutions.

23. Measure the physical water quality parameters and record as Day 4.

Test is complete. All surviving sac-fry are humanely euthanased at the completion of each test by immersion in a 10% solution of MS222, in accordance with Animal Experimentation Ethics regulations (NHMRC Animal Welfare Committee, 2003).

E.11 Randomisation

Randomness is an important component of the experimental design. Random distribution of sac-fry in test Petri dishes is achieved via steps 5–7. The Petri dishes are randomly assigned to positions on trays each day, meaning they will also have a random position in the incubator. Random numbers are obtained from a random number table or generator for each day of the test; a set of random numbers is not to be reused. When the sac-fry have to be observed, the Petri dishes can be sorted into replicate groups for greater convenience. This avoids the continual changing of glass pipettes by working through the water changes from a lower to a higher chemical concentration. At the end of the water changes the Petri dishes are again randomly placed on trays and returned to the incubator.

E.12 Avoiding bias

To avoid observer bias there should be at least two observers, however, in some instances this will not be possible. Each observer randomly selects a replicate group to record each day, and observations commence with the next highest chemical concentration to that which was first observed the previous day. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section E.13, should be used.

E.13 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Uranium (added as uranyl sulphate) is used in a concentration range from 1000–2000 ug/L. Synthetic water is used as diluent (Appendix A.E.6). The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to uranium. If the value falls outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

E.14 Acceptability of test data

The test data is considered acceptable if:

1. The recorded temperature of the incubator remains within the prescribed limits;
2. The mean mortality of the combined control does not exceed 20%;
3. The presence of fungus on the sac-fry does not exceed 20% in control animals;
4. The recorded pH is within the prescribed limits (usually ± 0.2 unit of Day 0 values for each test concentration);

5. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 27°C;
6. The conductivity for each test solution was within 10% of the values obtained on Day 0;
7. The result of reference toxicity testing is within the set limits.

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

E.15 Analysis of test data

The endpoints of the gudgeon sac-fry survival test are measured as the 96 h LC_{50}, the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC). The LC_{50} is the effective concentration giving 50% reduction in sac-fry survival over 96 h compared to the controls. This is calculated using Trimmed Spearman-Karber analysis (Hamilton et al 1977) or Maximum Likelihood Probit analysis (ToxCalc Version 5.0.23D, Tidepool Software). After testing the data for normality and homogeneity of variances, Dunnett's Multiple Comparison Test is used to determine which treatments are significantly different from one another. This information enables estimation of the LOEC and NOEC. Alternative statistical measures to the NOEC and LOEC can also be calculated. The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, is estimated using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, is estimated using the approach described by Ahsanullah and Williams (1991).

Appendix E

BTT-E – Purple-spotted gudgeon sac-fry survival test – Stock culture maintenance

A.E.1 Collection and acclimation of Purple-spotted gudgeon

Purple-spotted gudgeons (*Mogurnda mogurnda*) are collected from local waterways within the Magela Creek system of the Alligator Rivers Region, NT, Australia. Fish are captured either by baited fish traps or by fine meshed dip nets or seine nets, and are brought back to the aquaculture facilities at the Environmental Research Institute of the Supervising Scientist (***eriss***). Initially they are placed in either 144 L or 288 L aquaria, where the number of fish in each aquarium is determined by the size of the fish. Observations are then made for a nominal period to ascertain fish health and acclimation to laboratory conditions, and also to determine the sex of the fish based on physical appearance of the urinogenital papilla (females have a shorter bifurcated papilla and males display a curved elongated papilla). Once the sex has been determined, the fish are divided into breeding groups, consisting of usually one male and one to three females per aquarium. Further observations are then carried out to assess the breeding groups for fecundity, fertility and embryo hatchability to avoid any site-specific trait interfering with a test. The fish stocks are renewed at regular intervals to ensure genetics do not vary in laboratory compared with wild stock over time.

A.E.2 Aquaculture laboratory

Aquaria are set up in rows within a constant temperature, light controlled laboratory. Native aquarium plants (eg hornwort) are placed within each aquarium providing refuge. Six washed black plastic plant pots with a diameter of 23 cm are placed in the aquaria. Small stones are placed inside the pots to anchor them, and the opening of each pot is directed towards the front of the viewing area to assist observation. The pots provide a 'cave' refuge for the fish, and also a spawning surface.

Six to eight breeding aquaria are set up to produce sac-fry at any one time so that a toxicity test can be commenced when needed. The water temperature in the aquaria is maintained at 27 ± 1°C using chiller and heater elements within holding tanks in the roof cavity. Water flows continually through the operating aquaria to maintain optimal quality and health of the fish.

A.E.3 Fish feeding and aquaria maintenance

A.E.3.1 Fish feeding

Fish are fed daily on a varied diet consisting of 'commercial fish pellet' (Aristo Pet high protein fish pellets) supplemented with live food when possible (eg macroinvertebrates such as water boatmen etc), and 'pea and prawn puree' PPP (Appendix E.3.2). It has been observed that such a diet is adequate to provide sufficient nutrition to the breeding fish and enable the continuous production of embryos for weeks at a time. In addition, it has been observed that the quality of the water in the aquaria can be maintained at a higher level with less fouling when using such food. Live food can be collected and placed with the fish, allowing eating to continue *ad libitum*.

A.E.3.2 Preparation of 'Pea & Prawn Puree' (PPP)

PPP is prepared routinely in the laboratory to supplement the gudgeon's diet, and is modified from the Leggett and Merrick (1987) frozen food mixture recipe. The recipe requires: a mixture of 30% whole raw minced prawn (ensuring the prawns are obtained from an Australian source as imported prawns may contain viruses that can survive freezing); 30% minced, oil-less fish fillet; 40% raw minced peas, zucchini, spinach and pumpkin; and 100 g calcium ascorbate (non-acidic vitamin C) per 3 kilograms of finished product mince. All ingredients are roughly mixed using a domestic fine guage mincer. The PPP mixture is frozen raw into flat slabs in zip lock plastic bags. Pieces of the frozen PPP mixture are broken off the main slab, thawed and then fed to the fish in the size of small edible lumps.

A.E.3.2 Aquaria maintenance

The aquaria are cleaned on a fortnightly basis (or more frequently if required) using a wide mouth vacuum siphon. Leftover food, faeces and any other debris is removed. To ensure fish are not subject to undue stress, a quarter water change is performed, and the water replaced at ambient temperature. Cleaning the aquaria allows a larger volume of water to be exchanged, and this has been shown to stimulate courtship and subsequent spawning (D Wilson pers comm).

A.E.4 Courtship and spawning

Gudgeon breeding is variable, however it is possible to predict the approximate time a batch of eggs will be produced, based on careful observation of both behaviour and physical characteristics of a pair of fish (ie courtship behaviour accompanied by distinct golden colouration on the abdomen of the breeding female, and swelling and protrusion of both male and female papillae).

The gudgeons will select a spawning site (eg black pot, back of thermometer, rock, side of aquarium), and the female lays a batch of eggs while the male fertilises them. Each day prior to feeding, the aquaria are carefully observed for the presence of newly spawned eggs with the aid of a torch. The eggs are tubular in shape, have transparent cases, and are generally laid in circular patches of various sizes depending on the size of the breeding female. The egg batches range in size from 300−1000 eggs. The eggs are left in the aquarium to be guarded by the male parent fish for 24−60 h after being laid (careful observation is required to ensure the snail population in the aquarium does not get too high as they may eat the eggs). The eggs are then removed from the breeding aquarium and either kept and reared as future in-house breeding stock, or are placed in a 2 L beaker containing half parent aquarium water and half test diluent water and allowed to hatch under laboratory conditions for use in a toxicity test (see Appendix E.5 for more details).

If a breeding group ceases spawning, the fish can be swapped into different aquaria with different combinations of individuals. Alternatively, spawning can be delayed in an aquarium by placing a partition in it to isolate the sexes. After the partition is removed, it has been observed that spawning usually recommences within a week. If there is excessive disturbance or pedestrian traffic around the aquaria the aquaculture laboratory is closed off to all staff except those performing fish maintenance.

A.E.5 Isolation of Purple-spotted gudgeon sac-fry

When a batch of eggs is produced, they are left in the parent aquarium for 24−60 h allowing the male parent fish to guard them. Infrequently the eggs may be eaten before they can be removed, however, it is noted that this is the exception rather than the rule, and may be due to

a number of reasons such as the presence of excessive numbers of water mites (eg Suborder Oribatida), or microcrustacea in the breeding aquaria which invade and feed on the egg mass, or carnivorous aquatic snails (eg *Amerianna* sp). To reduce the numbers of such fauna, a small black-striped rainbowfish (*Melanotaenia nigrans*) can be placed in breeding aquaria and removed again prior to spawning.

After 24–60 h development in the parent aquarium, the developing embryos are carefully removed by placing the pot or rock etc, into a 2 L beaker containing half parent aquarium water and half diluent water, ensuring that the temperature of this water is ± 1°C of the parent aquarium water. If the eggs are laid on a surface such as the wall of a pot, eggs can be removed for observation by carefully sliding a glass cover slip under the egg mass and moving it forward until the edge has some eggs attached to it. The cover slip with the eggs is transferred to a Petri dish with enough water to cover them while observations are made under a binocular stereo microscope. If the developing embryos appear normal, the batch is then placed on a warming tray set at 27 ± 1°C in the laboratory to continue development. They are observed regularly for deformities, fungal growth, viability or water mites etc. An airstone is positioned beneath the egg batch such that a gentle stream of bubbles passes upward over the surface of the eggs, simulating the fanning action of the male parent over the eggs to keep fungal spores from settling. The beaker is loosely covered with Glad®Wrap to stop airborne contamination etc. Frequent daily observations are made, ensuring minimal disturbance until hatching occurs. Half volume water changes are performed using test diluent water to ensure fouling does not occur and to gradually acclimatise the fish to 100% diluent water. It takes approximately 10 h for sufficient eggs to hatch that are needed to start a test. The test must be commenced with sac-fry that are less than 10 hours old. After all the eggs have hatched (or at least sufficient numbers to enable a test to commence), they are carefully isolated into Petri dishes using a wide mouth glass Pasteur pipette with an internal diameter at least 2 mm. Sufficient sac-fry are placed in three Petri dishes so that there are enough for each replicate to be started (usually three replicates). Any damaged sac-fry are discarded and humanely euthanased by exposing them to a 1g L^{-1} solution of the anaesthetic MS222.

A.E.6 Preparation of synthetic water

1 Prepare the stock solutions (table A.E.1) in 1 L volumetrics with Milli-Q water. Transfer to clean 1 L plastic bottles and store at 4°C until required.

Note: these ingredients are stored at 4°C, and will require replacing at 18–24 month intervals.

2 Fill a 5 L volumetric flask with Milli-Q water and pour this into a clean 25 L plastic barrel designated for synthetic water preparation.

3 Add the appropriate amount of the 7 solutions (described below) to the partially filled 5 L volumetric flask. Make flask up to volume with Milli-Q and pour into the barrel.

4 Fill the 5 L flask twice more to make the volume in the barrel equal 20 L.

5 Aerate overnight to allow mixing and gaseous exchange.

6 Check pH after a minimum of 12 hours aeration and adjust pH to 6.0 ± 0.15 using 0.05 M H2SO4 or 0.05 M NaOH.

7 The water can be stored at 4°C for up to 2 weeks if required. The pH is to be checked before use to ensure it remains within range.

Table A.E.1 Method for preparation of synthetic water

	Ingredient	Stock Solution (g/L)	Media Solution
1	$NaHCO_3$	72.34	1 mL/20 L
2	$Al_2(SO_4)_3.6H_2O$	17.26	1 mL/20 L
3	$MgSO_4.7H_2O$	121.52	1 mL/20 L
4	$CaCl_2.2H_2O$	32.96	1 mL/20 L
5	KCl	14.09	1 mL/20 L
6	$FeCl_3.6H_2O$	10	1 mL/20 L
7	Trace Element Solution	In 1 L add:	0.5 mL/20 L
	$CuSO_4.5H_2O$	0.11	
	$ZnSO_4.7H_2O$	0.123	
	$Pb(NO_3)_2$	0.008	
	$MnSO_4.H_2O$	1.188	
	$UO_2SO_4.3H_2O$	0.007	

PROTOCOL SHEET

Project Name Djalkmara pre-release testing **Project Number:**

Test Number: 611E **Test Name:** Fry_Djalk_02 **Start Date:** 17/12/02

BTT: E-Gudgeon sac-fry survival test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	3000 mL	0 mL
B	0.3%		9 mL
C	1.0%		30 mL
D	3.2%		96 mL
E	10%		300 mL
F	32%		960 mL
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: 611E **Test Name:** Fry_Djalk_02

Toxicant: Djalkmara Billabong **Diluent:** Magela creekwater

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	As per lab manual	As per lab manual
Site	Dajlkmara pumping station	Georgetown
Transportation	22 L jerry cans	22 L jerry cans
Comments	Collected by XX	Collected by

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: Chemical weighed out by:

Balance used: Stock solution prepared by:

TEST DETAILS			
BTT	E		
Species:	*Mogurnda mogurnda*		
Start Date:	17/12/02		
Start Time:	10:00am		
Started by:	XX		
Incubator no:	Beige-1		
Temps OK?	Yes		
Test waters prepared by:	XX		
Supervisor:	XY		
Chem. analysis: **J/N,S/N & date** **Submitted by:**	NTEL – metals AGAL – TOC, alkalinity		
Departure from BTT / comments:			

FRESH water parameters

Test No: 611E	**Test:** Fry_Djalk_02	**Species:** *Mogurnda mogurnda*	
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong	
PH:	**Cond:**	**pH:**	**Cond:**

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**					**Observer / Date:**
0	pH											
	Cond											
	DO											
1	pH											
	Cond											
	DO											
2	pH											
	Cond											
	DO											
3	pH											
	Cond											
	DO											
4	pH											
	Cond											
	DO											
5	pH											
	Cond											
	DO											

QC: pH stays w/I ±0.2 unit of Day 0 values for each Conc.; Cond for each test soln is w/I 10% of Day 0 values; DO conc. >70% air saturation value for each conc.

24 hour OLD water parameters

Test No: 611E	**Test:** Fry_Djalk_02	**Species:** *M. mogurnda*
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**			**Observer/ Date:**
1	pH									
	Cond									
	DO									
2	pH									
	Cond									
	DO									
3	pH									
	Cond									
	DO									
4	pH									
	Cond									
	DO									
5	pH									
	Cond									
	DO									
6	pH									
	Cond									
	DO									

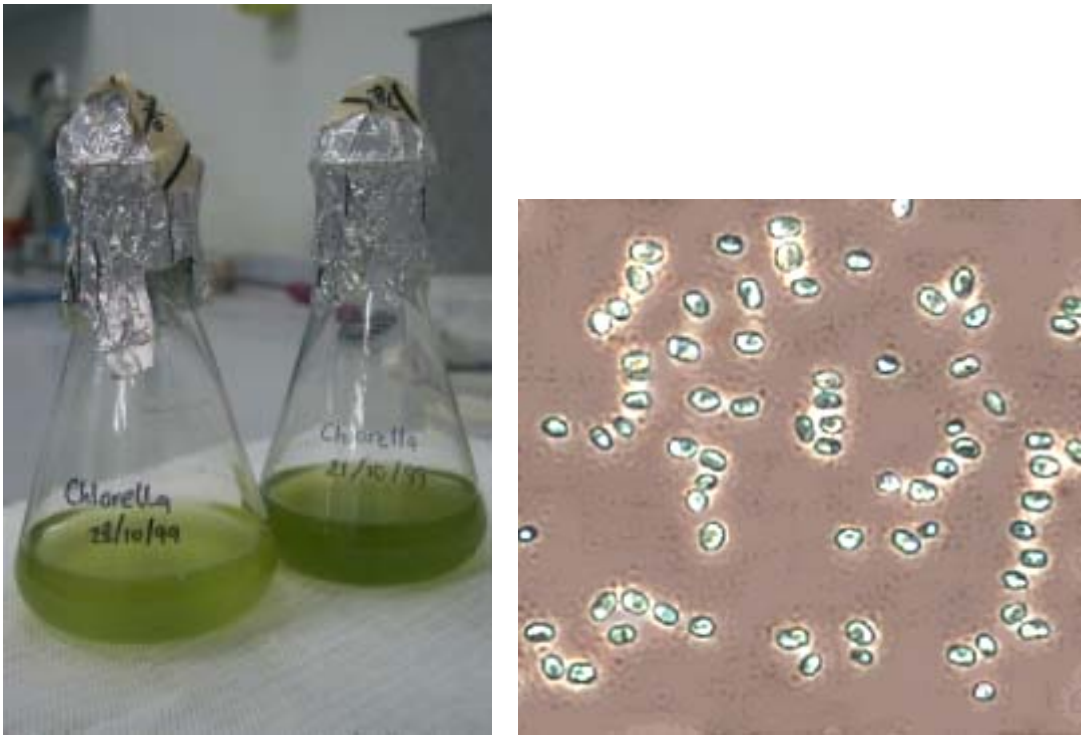
SAC-FRY SURVIVAL

Test No.: 611E

Test Name: Fry_Djalk_02 **Start Date:** 17/12/02 **Initial no./dish:** 10 **Replicate no. :** **1**

Day	Concentration / Observation	A Control	B 0.3%	C 1.0%	D 3.2%	E 10%	F 32%			
1	No. Alive									Observer/
	No. Dead									Date:
	No. fungused (ext)									
	Comments									Checked by:
2	No. Alive									Observer/
	No. Dead									Date:
	No. fungused (ext)									
	Comments									Checked by:
3	No. Alive									Observer/
	No. Dead									Date:
	No. fungused (ext)									
	Comments									Checked by:
4	No. Alive									Observer/
	No. Dead									Date:
	No. fungused (ext)									
	Comments									Checked by:

BTT-G: Algal growth inhibition test



Chlorella sp. Showing culture flasks and individual cells

BTT-G: Algal growth inhibition test

G.1 Objective

The objective of a test series (ie 3–4 definitive tests) is to determine the following response indicators for a specified chemical or whole effluent:

a) The No-Observed Effect Concentration (NOEC), which is the highest concentration at which algal growth is not statistically different ($P \leq 0.05$) from that of the control algae. This can be compared to the 10% bounded effect concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk, 1993);

b) The Lowest-Observed Effect Concentration (LOEC), which is the lowest concentration at which algal growth is significantly different ($P \leq 0.05$) from the control. This can be compared to the Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response becomes significantly ($P \leq 0.05$) lower than that of the control (Ahsanullah & Williams 1991);

c) The median effect concentration (IC_{50}), is the concentration of chemical in solution that is estimated to be effective in producing a sublethal response in 50% of test organisms This is measured as the 50% inhibition on the growth of test algae (*Chlorella* sp.) over 72 h.

G.2 Principle of the test

Exponentially growing cells of *Chlorella* sp. are exposed to various concentrations of a toxicant over several generations under defined conditions. The test is conducted over 72 h with cell counts undertaken at both 48 and 72 h. From these counts, cell division rates are calculated. A test substance is considered toxic when a statistically significant ($P \leq 0.05$) concentration-dependent inhibition of algal growth occurs. Development of this method is described by Franklin et al (1998).

G.3 Test organism

The unicellular freshwater green alga *Chlorella* sp. (Chlorophyceae) was isolated from surface water collected within Kakadu National Park (Padovan 1991) and is maintained at the ***eriss*** facility as described in Appendix G. Original test algae were collected from Magela Creek (Georgetown Billabong) by Armando Padovan in 1991.

G.4 Dilution water

Depending on the aim of the test, either synthetic softwater or natural Magela Creek water is used as the test diluent.

G.4.1 Synthetic water

'Synthetic' water simulates the inorganic composition of water from Magela Creek and many other sandy braided streams from northern Australia during the Wet season. This water is characteristically very soft, slightly acidic and has a low buffering and complexation capacity. These qualities make synthetic softwater particularly useful in providing a worst case toxic scenario, as under these conditions many toxicants (particularly metals) are highly available

to the test organism. The synthetic water is prepared by adding analytical grade reagents to Milli-Q water (< 1 µS cm^{-1}) (table G.1) in acid-washed polyethylene containers (see appendix A.G.3). This is done as close as practical to the start of the test. The pH of the test water is adjusted to the required level (in this case 6.0 ± 0.15 at 27 ± 1°C) with 0.02 M HNO_3 or 0.0125 M NaOH. The test water is stored in sealed polyethylene containers and refrigerated (4°C) until use.

Table G.1 Mean nominal composition of synthetic water

Physico-chemical parameter	Total concentration
pH	6.0 ± 0.15[a]
Temperature (°C)	27 ± 1[a]
Na	1.00 mg L^{-1}
K	0.37 mg L^{-1}
Ca	0.45 mg L^{-1}
Mg	0.60 mg L^{-1}
Cl	2.32 mg L^{-1}
SO_4	3.12 mg L^{-1}
HCO_3	2.63 mg L^{-1}
NO_3	0.07 µg L^{-1}
Fe	100 µg L^{-1}
Al	70 µg L^{-1}
Mn	9.7 µg L^{-1}
U	0.10 µg L^{-1}
Cu	0.70 µg L^{-1}
Zn	0.70 µg L^{-1}
Pb	0.12 µg L^{-1}

G.4.2 Natural stream water

Natural stream water is the receiving water taken upstream of the waste water discharge outlet (eg Magela Creek upstream of Georgetown pumping station latitude 12° 40' 28" longitude 132° 55' 52" – Wet season *or* Bowerbird billabong latitude 12° 46' 15", longitude 133° 02' 20" – Dry season). It should be collected in acid-washed plastic containers as close as is practicable to the start of the test, ie afternoon prior to test commencement, and stored at 4°C overnight. Within 48 hours of collection, the required amount of water for the test should be filtered through a fine pore size filter (eg Whatman No 42, 2.5 µm) capable of removing 'wild' zooplankton. The water should be stored in covered polyethylene containers at 4°C for a maximum period of three weeks.

G.5 Stock solutions

G.5.1 Chemical solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in an acid-washed, plastic container and refrigerated (4°C). The source of the stock solution (eg date of preparation, by whom) is marked on the bottle

and on an information sheet. Immediately prior to use for test water preparation the stock solution is allowed to equilibrate to room temperature.

G.5.2 Whole effluent solutions

Whole effluent stock solutions are collected from the designated site as close as is practicable to the start of the test, in an acid-washed plastic or glass container (depending on the chemical properties of the effluent). The sample should be kept sealed, clearly labelled and refrigerated at 4°C until required for test commencement.

G.6 Test solutions

Test solution preparation for algal tests involves modification of diluent water in order to promote good algal growth. To make 5 L of modified stream water, 45 mL of 2.21 g/L $NaNO_3$ (14.5 mg/L NO_3), 4.5 mL of 0.22g/L KH_2PO_4 (0.14 mg/L PO_4) and 10 mL of 130.15 g/L HEPES (*N*-2hydroxypiperazine-*N*'-2ethanesulfonic acid) buffer (260 mg/L) is combined with filtered stream water in a 5 L volumetric flask. Stauber et al (1994) found this concentration of nitrate and phosphate to be the only nutrients required to maintain exponential growth over a 72 h period for the temperate freshwater alga *Chlorella protothecoides*. A 500 mL batch of solution is prepared for each treatment by adding the appropriate volume of stock solution to modified stream water in a volumetric flask. Aliquots (50 mL) of test solution are dispensed into each of three test containers and a sample bottle to be sent to an external laboratory for chemical analysis.

G.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the alga are placed, or the alga themselves, should be chemically inert

G.7.1 Container preparation

All containers (ie bottles, Petri dishes and lids etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 μS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least 3 times with Millipore water (< 1 μS cm^{-1});
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with appropriate diluent or control water. Other equipment should be rinsed thoroughly with Millipore water (< 1 μS cm^{-1}) before use.

G.7.2 Glassware preparation

Erlenmeyer flasks (250 mL) used exclusively in the algal toxicity test are soaked and washed as described in Section G.7.1. The flasks are coated with a silanising solution such as Coatasil (Ajax), dried in the fume cupboard for 24 h and acid-washed again immediately before use. This silanising step is essential to reduce toxicant adsorption to the walls of the glass flasks (which may reduce the availability and subsequent toxicity of the test chemical to the algae). Silanisation of the flasks should be redone periodically as continual washing gradually removes the surface coating.

G.7.3 Temperature control

Tests are conducted at 29 ± 1^{o}C using a constant temperature growth cabinet. During observation, test containers are removed from the growth cabinet for the minimum amount of time possible to avoid fluctuations in test solution temperature.

G.7.4 Photoperiod control

Tests are conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity ranges between 100–150 μmol m^{-2} s^{-1} (Photosynthetically Active Radiation) and is checked periodically using a light meter.

G.7.5 Equipment

- Haemocytometer or electronic particle counter for algal cell enumeration
- Haemocytometer cover glasses
- Glass tissue homogeniser (hand-held – 15 mL) with teflon pestle
- Automatic partile counter (eg Coulter Multisizer)
- Isoton for electronic particle counting
- Disposable plastic counting cups for the electronic particle counter
- Microscope with phase contrast providing 200 × magnification
- Environmental chamber or growth room with light boxes and temperature recorder
- Light meter
- Centrifuge – 4 × 500 mL capacity with swing out buckets
- Plastic centrifuge tubes – 50 mL capacity
- Centrifuge tube racks (perspex or equivalent)
- Vortex mixer
- Milli-Q water purification system or equivalent
- Refrigerator for storage of test and stock solutions
- Borosilicate glass 200 mL Erlenmeyer flasks with aluminium caps
- A-grade glass volumetric flasks
- pH meter, pH probe and pH buffer solutions of 7.00 and 4.00
- Magnetic stirrer and stirrer bars

- Filter apparatus – 5 L plastic Buchner funnel, 5 L flask, vacuum pump and tubing
- Membrane filters – 2.5 μm pore size (Whatman No 42)
- Automatic adjustable pipettes (5 μL to 5 mL)
- Disposable microlitre pipette tips
- Polyethylene storage containers (1 L to 10 L)
- Analytical balance and weigh boats
- Glass pasteur pipettes
- Chemicals and reagents

G.8 Test environment

The preparation and storage of test solutions, culturing of test algae, and conducting tests should be carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

G.9 Data recording

The number of algal cells in each test treatment are counted at 48 and 72 h after test commencement (when t = 0 h). Observations made at the end of the 48 h period are designated as Day 2 observations and at the end of the 72 h period as Day 3 observations. The pH of one replicate is measured and recorded at the beginning and end of the test.

G.10 Test procedure

Day 0 (ie starting day of test t = 0 h)

1. An inoculum of algal cells consisting of exponentially growing *Chlorella* sp. cells harvested from a 4–5 d old stock culture is prepared 2–3 h before the start of the test. The algal cells are centrifuged in 50 mL plastic centrifuge tubes at 2500 revolutions per minute (rpm) at 20°C in a Heraeaus Megafuge refrigerated centrifuge for 7 min. The nutrient medium (supernatant) is decanted and the cell pellet re-suspended in about 30 mL of Milli-Q water by gentle vortexing. The centrifugation and washing procedure is repeated three times to remove the high nutrient culture medium, which would otherwise ameliorate toxicity due to its ability to strongly complex trace metals (Stauber & Florence 1989). The cell pellet is finally re-suspended in about 15 mL of Milli-Q water.
2. A hand-held glass tissue grinder with teflon pestle is used to separate clumps in the re-suspended cells. The cell density of the suspension is determined by diluting a 50 μL sample to 50 mL and counting manually using a microscope and haemocytometer. From this the volume of algae inoculum required to give a starting cell density of 2–4 x 10^4 cells mL^{-1} in the test flask can be determined.
3. Test solutions are prepared as described in section G.6 and left at room temperature.
4. Test flasks are inoculated with 2–4 × 10^4 cells mL^{-1} of prewashed *Chlorella* sp. cells. One flask has no algae added. This serves as a background correction for the automatic particle counts.

5. The test flasks are placed randomly in an environmental cabinet at 29 ± 1°C on a 12:12 h light/dark cycle (Phillips TL 40W cool white fluorescent lighting, 100–150 μmol m^{-2} s^{-1}).

Days 1–3

6. Each flask is gently agitated by hand twice daily throughout the test to avoid gas limitation. This is done by swirling the solution approximately six times in the clockwise direction and six times in the anti-clockwise direction.

7. The cell density in each flask is determined at both 48 and 72 h by using either an automatic particle counter, such as a Coulter Multisizer II, or manually using a haemocytometer and a microscope.

Electronic cell enumeration

A Coulter Multisizer II Particle Analyser with a 70 μm aperture is used in the narrow mode, with window settings ranging from 1.45–9.36. Before counting, the test flasks are well mixed by swirling the solution six times in a clockwise direction and six times in an anti-clockwise direction. A 2.5 mL aliquot of cells is immediately taken from each flask and diluted to 10 mL in a volumetric flask with Isoton II electrolyte solution (Coulter Electronics Pty Ltd, Brookvale, Australia). This dilution in Isoton is necessary to provide sufficient electrolytes for electronic particle counting. Samples are homogenised gently in a tissue grinder to break cell clumps prior to counting. The sample is poured directly into a plastic counting cup and the algae is counted with the particle counter. Four 100 μL aliquots are counted for each flask and the mean count ± 2 standard deviations (SD) determined. A background count (1:4 dilution with Isoton) from a test flask containing no algal cells is also determined. This 'Coulter blank', arising from small particles other than algal cells, is then subtracted from the mean algal cell count for each flask.

Manual cell enumeration

Algal cells may be counted using a phase contrast microscope and a haemocytometer. This method is considerably slower than the electronic counting method, but permits the direct examination of the cell morphology.

Sub-samples from each flask (1–2 mL) are taken and homogenised in a tissue grinder to break cell clumps. No dilution with seawater or background count correction is required. At least 200 individual cells should be counted per sample to maintain counting errors of <20%.

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

G.11 Randomisation

Randomness is an important component of the experimental design. On each day a new set of random numbers must be used for the position of each flask in the incubator for the next 24 h period. The flasks are repositioned each morning after being swirled to aid gas exchange.

G.12 Avoiding bias

To avoid observer bias there should be at least two observers, however, in some instances this will not be possible. Each observer alternates between preparing samples for counting and operating the automatic particle counter. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates

in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section G.14, should be used.

G.13 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Uranium (added as uranyl sulphate) is used in a concentration range from 5–320 ug/L. Synthetic water is used as diluent (appendix A.G.3). The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to uranium. If the value falls outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

G.14 Acceptability of test data

The test data is considered acceptable if:

1. The recorded temperature of the growth chamber remains within the prescribed limits;
2. The growth rate of the control algae is within the range 1.4 ± 0.3 doublings day^{-1}
3. There is < 20% variability in the control growth rate;
4. The recorded pH are within the prescribed limits;
5. The result of reference toxicity testing is within the set limits.

G.15 Analysis of test data

The growth rate (cell division rate) of algae in each flask over 72 h is calculated using linear regression analysis. A regression line is plotted for $\log_{10}$ 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. Daily doubling times are calculated by multiplying this value $\mu \times 24 \times 3.32$ (constant). A test acceptability measure (mean cell division rate in controls ± 2 SD) has been derived from the results of optimisation tests (Franklin 1998). Test results are acceptable if the control growth rates were within this range (1.40 ± 0.3 doublings d^{-1}). Growth rates of the treated flasks are presented as a percentage of the control growth rate. A concentration–response curve is then fitted to the data by plotting the percentage control growth rates vs the measured toxicant concentrations.

The endpoints of the algal growth inhibition test are measured as the 72 h IC_{50} and the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC). The IC_{50} is the effective concentration giving 50% reduction in algal growth rate over 72 h compared to the controls. Linear interpolation can be used to calculate IC values. After testing the data for normality and homogeneity of variances, Dunnett's Multiple Comparison Test is used to determine which treatments are significantly different from one another. This information enables estimation of the LOEC and NOEC. Alternative statistical measures to the NOEC and LOEC can also be calculated. The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, is estimated using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, is estimated using the approach described by Ahsanullah and Williams (1991).

Appendix G

BTT-G: Algal growth inhibition test – Stock culture maintenance

The alga is cultured axenically in a modified MBL medium (Stein 1973). Ten stock nutrient solutions are prepared in A-grade volumetric flasks using analytical grade reagents and Milli-Q water (table A.G.1).

To prepare enough liquid media to maintain the algae for 5 weeks, add 5 mL of stock 1 and 1 mL of stocks 2–10 to 1 L of Milli-Q water in a volumetric flask and mix thoroughly. Adjust the media to pH 7.2 ± 0.1 using 10% HCl (approx. 25–30 drops). Dispense 100 mL of media into ten 250 mL conical flasks, insert cotton wool bungs and cover with aluminium foil. Autoclave the medium at 121°C and 120 kPa for 20 min. Allow to cool overnight for re-equilibration of CO_2. The final concentrations of the macro and micronutrients in the liquid growth medium are shown in table 2.

Aseptically transfer 2 mL of the algal culture to the liquid growth medium in a 250 mL Erlenmeyer flask using a disposable sterile glass pipette. Incubate the culture at 29 ± 1°C on a 12:12 h light/dark cycle (Phillips TL 40W cool white fluorescent lighting, 75 μmol m^{-2} s^{-1}). No shaking is necessary. The culture should be renewed every week to ensure a regular supply of exponentially growing cells for the toxicity test.

Routine microscope examination of the algal stock culture, using a phase contrast microscope, should be carried out routinely to ensure good cell morphology and the absence of contaminants.

Table A.G.1 Stock nutrient solutions for the maintenance of *Chlorella* sp. (MBL medium)

Stock ingredients	Stock concentration	Volume added to media	Media concentration
1. Tris buffer	100 g/L	5 mL/L	500 mg/L Tris
2. $NaNO_3$	85.24 g/L	1 mL/L	85 mg/L $NaNO_3$
3. $CaCl_2.2H_20$	36.76 g/L	1 mL/L	10 mg/L Ca^{2+}
4. $MgSO_4.7H_20$	36.97 g/L	1 mL/L	6.6 mg/L Mg^{2+}
5. $NaHCO_3$	12.6 g/L	1 mL/L	13 mg/L $NaHCO_3$
6. K_2HPO_4	8.72 g/L	1 mL/L	8.7 mg/L K_2HPO_4
7. Na_2EDTA	4.36 g/L	1 mL/L	4.4 mg/L Na_2EDTA
8. $FeCl_3.6H_20$	0.727 g/L	1 mL/L	152 μg/L Fe^{3+}
Trace metals		1 mL/L	
$CoCl_2.6H_2O$	10 mg/L		2.2 μg/L Co^{2+}
$CuSO_4.5H_2O$	9 mg/L		2.3 μg/L Cu^{2+}
$NaSiO_3.5H_2O$	7 mg/L		1.0 μg/L Si^{2+}
$MnCl_2.4H_2O$	180 mg/L		50 μg/L Mn^{2+}
$ZnSO_4.7H_2O$	22 mg/L		5.0 μg/L Zn^{2+}
Vitamins		1 mL/L	
Pipette 2.5 mL of biotin stock (0.05 g/500 mL) and 2.5 mL of vitamin B_{12} stock (0.025 g/250 mL) into a 250 mL volumetric flask containing 0.05 g thiamine. Make up to volume with Milli-Q water.			1 μg/L biotin 1 μg/L vitamin B_{12} 200 μg/L thiamine

A.G.1 Preparation of synthetic water

1. Prepare the stock solutions (table A.G.2) in 1 L volumetrics with Milli-Q water. Transfer to clean 1 L plastic bottles and store at 4°C until required.

Note: these ingredients are stored at 4°C, and will require replacing at 18–24 month intervals.

2. Fill a 5 L volumetric flask with Milli-Q water and pour this into a clean 25 L plastic barrel designated for synthetic water preparation.
3. Add the appropriate amount of the 7 solutions (described below) to the partially filled 5 L volumetric flask. Make flask up to volume with Milli-Q and pour into the barrel.

Table A.G.2 Method for preparation of synthetic water

	Ingredient	Stock Solution (g/L)	Media Solution
1	$NaHCO_3$	72.34L	1 mL/20 L
2	$Al_2(SO_4)_3.6H_2O$	17.26	1 mL/20 L
3	$MgSO_4.7H_2O$	121.52	1 mL/20 L
4	$CaCl_2.2H_2O$	32.96	1 mL/20 L
5	KCl	14.09	1 mL/20 L
6	$FeCl_3.6H_2O$	10	1 mL/20 L
7	Trace Element Solution	In 1 L add:	0.5 mL/20 L
	$CuSO_4.5H_2O$	0.11	
	$ZnSO_4.7H_2O$	0.123	
	$Pb(NO_3)_2$	0.008	
	$MnSO_4.H_2O$	1.188	
	$UO_2SO_4.3H_2O$	0.007	

4. Fill the 5 L flask twice more to make the volume in the barrel equal 20 L.
5. Aerate overnight to allow mixing and gaseous exchange.
6. Check pH after a minimum of 12 hours aeration and adjust pH to 6.0 ± 0.15 using 0.05 M H_2SO_4 or 0.05 M NaOH.
7. The water can be stored at 4°C for up to 2 weeks if required. The pH is to be checked before use to ensure it remains within range.

PROTOCOL SHEET

Project Name Djalkmara pre-release testing **Project Number:**

Test Number: 611G **Test Name:** Algae_Djalk_02 **Start Date:** 17/12/02

BTT: G-Algal growth inhibition test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	3000 mL	0 mL
B	0.3%		9 mL
C	1.0%		30 mL
D	3.2%		96 mL
E	10%		300 mL
F	32%		960 mL
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: 611G **Test Name:** Algae_Djalk_02

Toxicant: Djalkmara Billabong **Diluent:** Magela creekwater

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	As per lab manual	As per lab manual
Site	Djalkmara pumping station	Georgetown
Transportation	22 L jerry cans	22 L jerry cans
Comments	Collected by XX	Collected by XX

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: Chemical weighed out by:

Balance used: Stock solution prepared by:

TEST DETAILS			
BTT	G		
Species:	*Chlorella* sp.		
Start Date:	17/12/02		
Start Time:	10:00am		
Started by:	XX		
Incubator no:	Beige-1		
Temps OK?	Yes		
Test waters prepared by:	XX		
Supervisor:	XY		
Chem. analysis: **J/N,S/N & date** **Submitted by:**	NTEL – metals AGAL – TOC, alkalinity		
Departure from BTT / comments:			

FRESH water parameters

Test No: 611G	**Test:** Alage_Djalk_02	**Species:** *Chlorella* sp.	
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong	
PH:	**Cond:**	**pH:**	**Cond:**

Conc. / Day		A **Control**	B **0.3%**	C **1.0%**	D **3.2%**	E **10%**	F **32%**					**Observer / Date:**
0	pH											
	Cond											
1	pH											
	Cond											
2	pH											
	Cond											
3	pH											
	Cond											
5	pH											
	Cond											
6	pH											
	Cond											

QC: pH stays w/I ±0.2 unit of Day 0 values for each Conc.; Cond for each test soln is w/I 10% of Day 0 values; DO conc. >70% air saturation value for each conc.

24 hour OLD water parameters

Test No: 611G	**Test:** Algae_Djalk_02	**Species:** *Chlorella* sp.
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**			**Observer/ Date:**
	pH									
1	Cond									
	pH									
2	Cond									
	pH									
3	Cond									
	pH									
4	Cond									
	pH									
5	Cond									
	pH									
6	Cond									

Coulter Counter Sheet

Date: 17/12/02 Day: 3 Temperature:

Test No.: 611G Test: Alg_Djalk_02

Species: *Chlorella sp* Toxicant: Djalkmara Billabong

Flask No.: 1 Concentration: Dilution: 1:4

TIME (sec)	COUNTS	%	SETTING (μm)
No. Cells per mL:			

Flask No.: 2 Concentration: Dilution: 1:4

TIME (sec)	COUNTS	%	SETTING (μm)
No. Cells per mL:			

Flask No.: 3 Concentration: Dilution: 1:4

TIME (sec)	COUNTS	%	SETTING (μm)
No. Cells per mL:			

BTT-I: Cladoceran immobilisation test

Moinodaphnia macleayi King showing juvenile at left, and adult female with eyed young in the brood pouch on the right

BTT-I: Cladoceran immobilisation test

I.1 Objective

The objective of a test series (ie 3–4 definitive tests) is to determine the concentrations of a specified chemical or whole effluent that shows:

a) The No Observed Effect Concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the 10% Bounded Effect Concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk 1993);

b) The Lowest Observed Effect Concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response becomes significantly ($P \leq 0.05$) lower than that of the control (Ahsanullah & Williams 1991), and;

c) The median effect concentration (EC_{50}), is the concentration of chemical in solution that is estimated to be effective in producing a sublethal response in 50% of test organisms. For this test it is measured as the EC_{50} on the immobilisation and survival of test cladocera over 48 h.

I.2 Principle of the test

Asexually-reproducing female test Cladocera are immersed in a range of concentrations of the test water to be assessed under defined conditions. These females are *not* transferred daily to fresh solutions of the same concentration. Each day, observations are made on the survival of each female and whether immobilised or not. Each female must be accounted for as alive, immobilised, dead or missing, rather than assuming missing animals are dead. The test is terminated after 48 hours. The method is based on the *Daphnia* sp., acute immobilisation test developed by the OECD (1999).

I.3 Test organism

The test species is *Moinodaphnia macleayi* King (Crustacea, Cladocera) commonly known as a water-flea or cladoceran (Smirnov & Timms 1983). The test water fleas should be less than six hours old at the start of the test (ie neonates), and are obtained from laboratory stock cultures as described in Appendix D.1. All the test water fleas must be asexually-reproducing (parthenogenetic) females. Asexual reproduction is a characteristic of this type of organism under optimal environmental conditions. Test water fleas should be free from overt disease or gross morphological deformity. Original test cladocerans were collected from Magela Creek, with recent cultures restocked from Bowerbird Billabong in September 2002.

I.4 Dilution water

There are two diluent water types which are routinely used – natural stream water and dechlorinated filtered Darwin tap water (which can be used as an analogue of low conductivity, low mineralised soft water). The aim of the test will determine which water type is used.

I.4.1 Natural stream water

Natural stream water is the receiving water taken upstream of the waste water discharge outlet (eg Magela Creek upstream of Georgetown pumping station (latitude 12° 40' 28", longitude 132° 55' 52") – Wet season *or* Bowerbird Billabong (latitude 12° 46' 15", longitude 133° 02' 20") – Dry season). It should be collected in acid-washed plastic containers as close as is practicable to the start of the test, ie afternoon prior to test commencement, and stored at 4°C overnight. Within 48 hours of collection, the required amount of water for the test should be filtered through a fine pore size filter (eg Whatman No 42, 2.5 μm) capable of removing 'wild' zooplankton. The water should be stored in covered plastic containers at 4°C for a maximum period of three weeks.

I.4.2 Filtered Darwin tap water

Darwin tap water is low conductivity, low mineralised good quality soft water. At the ***eriss*** laboratory, water travels from the mains supply predominantly through PVC pipe to two activated carbon filter units. After passing through these the water is then pumped into the roof cavity and is held in two temperature controlled (27 ± 1°C) holding tanks. When water is required for use in culturing or as diluent water for testing, it travels through insulated PVC plastic pipes from the holding tanks to the aquaculture laboratory. General water quality parameters are routinely measured, while water chemistry samples are regularly collected for analysis of major anions and cations.

I.5 Stock solutions

I.5.1 Chemical solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in an acid-washed, polyethylene container and refrigerated (4^oC). The source of the stock solution (eg date of preparation, by whom) is marked on the bottle and on an information sheet. Immediately prior to use for test water preparation the stock solution is allowed to equilibrate to room temperature.

I.5.2 Whole effluent solutions

Whole effluent stock solutions are collected from the designated site as close as is practicable to the start of the test, in acid-washed plastic or glass containerers (depending on the chemical properties of the effluent). The sample should be kept sealed, clearly labelled and refrigerated at 4°C until required for test commencement.

I.6 Test solutions

Test solutions are prepared by diluting a stock solution with diluent water. The pH is then adjusted if necessary, using 0.02 M HNO_3 or 0.0125 M NaOH. Test solution concentrations are determined from the results of range-finding studies. Test solutions are prepared in bulk at the start of a test in 2–5 L polyethylene or glass screw-topped containers and refrigerated (4^oC) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies significantly when stored for the test period.

I.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the cladocerans are placed, or the cladocerans themselves, should be chemically inert.

I.7.1 Container preparation

All containers (ie bottles, plastic vials etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 μS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least 3 times with Millipore water (< 1 μS cm^{-1});
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with appropriate diluent or control water. Other equipment should be rinsed thoroughly with Milli-Q water (< 1 μS cm^{-1}) before use.

I.7.2 Temperature control

Tests are conducted at 27 ± 1°C using a constant temperature incubator. When removed from the incubator for observation, the temperature of the test containers are maintained at 27 ± 1°C by the use of warming trays on the microscope bench.

I.7.3 Photoperiod control

Tests are conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie 30–100 μmol m^{-2} s^{-1}).

I.7.4 Equipment

- Light-tight constant temperature incubator
- Maximum-minimum thermometer
- Milli-Q water purification system or equivalent
- A-grade glass volumetric flasks
- Chemicals and reagents
- Analytical balance and weigh boats
- Filter apparatus – 5 L plastic Buchner funnel, 5 L glass flask with side arm, vacuum pump and tubing

- Membrane filters – 2.5 μm pore size (Whatman No 42)
- Magnetic stirrer and stirrer bars
- 5 L polyethylene containers (to hold treatment solutions, one per solution)
- Refrigerator for storage of test and stock solutions
- 250 mL volume polypropylene test containers and lids with air holes (ie 24 for an eight treatment test)
- 150 mL tall form polypropylene containers for water parameter measurement (ie 16 for an eight treatment test)
- pH meter, electrical conductivity EC meter and dissolved oxygen meter and appropriate calibration standards
- Binocular dissecting microscope with bright field/dark field illumination
- Automatic 0–50 mL dispenser
- Three or four clear perspex trays with position numbers marked 1–24, each capable of holding eight 250 mL test vials (for an eight treatment test)
- Laboratory warming trays, set at 27 ± 1^{o}C, capable of accommodating the clear plastic trays
- Random number generator
- Plastic tray to hold 150 mL containers
- Pasteur pipettes, with internal tip diameter of ~ 1 mm
- Algae and Fermented Food with Vitamins (FFV) for feeding the water fleas
- Automatic adjustable pipettes (5 μL to 5 mL) and disposable pipetter tips
- Data sheets
- 50–200mL acid-washed bottles (glass or polyethylene depending on toxicant) with plastic screw caps, for analytical chemistry samples.

I.8 Test environment

The preparation and storage of test solutions, culture of test cladocerans, and conduct of tests should be carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

I.9 Data recording

Test animals are observed and data recorded at 24 h intervals after the commencement of the test (when t = 0 h). Observations made at the end of the first 24 h period are designated as Day 0 observations; at the end of the second 24 h period, Day 1 observations etc. Water quality parameters are measured and adjusted (where appropriate) and recorded at the beginning and end of each 24 h period, and are designated as Fresh Water Day 0, 24 h old Water Day 1, respectively, and so forth during the test.

I.10 Test procedure

Day 0 (ie start day of test)

1. Prepare the test solutions (as outlined in Section I.6) and equilibrate to 27 ± 1°C.
2. Prior to the expected commencement time of the test, isolate 24 mature female cladocerans with eyed young visible in their brood chamber (or with their second brood if neonates less than 6 h old are already present) randomly into three glass crystallising dishes containing diluent water (one dish for each of three replicates). Place the crystallising dishes on a warming tray to maintain at test temperature. The test should commence within six hours of the birth of brood two neonates.
3. To each 250 mL test vial add 150 µL FFV (Fermented Food with Vitamins) and the required amount of algae (6×10^6 cells; see Appendix I.6). Then immediately dispense 150 mL aliquots of each test concentration (normally 8) into each of three appropriately labelled replicate 250 mL vials (ie 3 × 150 mL for each concentration) and allow to equilibrate to incubator temperature (27 ± 1°C). Arrange vials in replicate groups on the clear perspex vial trays. Also dispense and cap a sample of each test solution (50–100 mL with food and algae added at the correct ratio) into appropriately labelled 150 mL containers for the measurement of 'fresh' water quality parameters (pH, conductivity and dissolved oxygen).
4. Using a microscope and Pasteur pipette with tip < 1 mm, pick out one test neonate from the isolated stock, and drop gently into control replicate 1 without touching the test solution.
5. Repeat for the remaining test concentrations of relicate one, working up in concentration and ending with the highest concentration.
6. Discard the used pipette and obtain a clean one if the test solution was touched at any point.
7. Repeat steps 4–6 for the other vials until all vials for each concentration contain 10 neonates.
8. Observe each dish under the microscope to ensure that there are 10 neonates in each vial, and replace any neonates that are damaged in any way. If damaged, replace immediately with 'suitable test neonates' using a new pipette.
9. Repeat steps 4–8 for the remaining two replicate groups.

Note: More than one person can distribute test neonates simultaneously, with the distribution appropriately split into replicate groups.

10. When 10 neonates have been distributed into each of the test containers, cover with plastic caps with air holes and place them in the random order for that day (see Section I.11).
11. Place the 3 trays in the incubator.
12. Completion of this stage constitutes the start of the test (time = 0 hours) and is recorded on the information sheet in the test folder.
13. Measure water quality parameters ie pH, conductivity and dissolved oxygen.

Note: Whenever test containers are removed from the incubator maintain them at 27°C (eg by placing them on a warming tray).

Day 1

14. 24 hours after the commencement of the test, remove trays from incubator and sort the test vials into numerical order for each concentration group.
15. For each concentration group, using a microscope make the following observations and record results:
    a) Record whether the test flea is alive, dead or missing;
    b) Make any other observations which suggest that the water fleas are not developing normally eg haemorrhaging, small size etc.
16. Observations are recorded as Day 1 observations.
17. To avoid observer bias, each day select a different concentration group to observe first.
18. When test water fleas from all eight concentration groups have been observed, place vials into the random order for that day, and place trays into the incubator in the random order for that day (see Section I.11).

Day 2 (ie 48 h end of test)

19. Repeat steps 14–16.
20. Measure the water quality parameters for the 24-hour-old water and record as Day 2 (depending on which day the test finishes).

Test is complete at 48 h after commencement.

I.11 Randomisation

On each day a new set of random numbers must be used to assign the position of each vial on each plastic tray, and the position of each plastic tray in the incubator every 24 h. Randomness is an important component of the experimental design. Random distribution of cladocerans is achieved via steps 4–6. The vials are randomly assigned to positions on trays each day and they will also have a random position in the incubator. Random numbers are obtained from a random number table or generator for each day of the test; a set of random numbers is not to be reused. When the water fleas have to be observed, the vials and trays can be sorted into numerical order for greater convenience. This avoids the continual changing of glass pipettes by working through the water changes from a lower to a higher chemical concentration. At the end of the water changes the Petri dishes are again randomly placed on trays and returned to the incubator.

I.12 Avoiding bias

To avoid operator bias there should be at least two people present to carry out the test when possible. The two operators alternate the concentrations they observe each day. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section I.13, should be used.

I.13 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Uranium (added as uranyl sulphate) is used in a concentration range from 150–450 ug/L. Natural Magela Creek water is used as diluent. The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to uranium. If the value falls outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

I.14 Acceptability of test data

The data set of the test (ie all data from each group) is considered acceptable if:

1. The recorded temperature of the incubator remains within the prescribed limits;
2. 90% or more of the test control cladocera are alive;
3. The result of reference toxicity testing is within the set limits.

The data of each test group is considered acceptable if:

1. The recorded pH is within the prescribed limits (usually ± 0.2 unit of Day 0 values for each test concentration);
2. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 27°C;
3. The conductivity for each test solution was within 10% of the values obtained on Day 0;
4. No more than 20% of parental cladocerans are reported missing in any treatment (except if all other cladocerans are dead in that group).

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

I.15 Analysis of test data

Statistical endpoints for most tests are determined using the data analysis package, ToxCalc. Version 5.0.23D (Tidepool Software). This package enables all required toxicity indices to be calculated using parametric and non-parametric statistical methods. Analysis of the data was performed using a range of methods including Steels Many-One Rank Test (non-parametric) and Dunnett's Multiple Comparison Test (parametric). LOEC and NOEC values were obtained using hypothesis testing ($p<0.05$) and point estimates are calculated using Maximum Likelihood Probit Analysis for Acute Immobilisation tests. Point estimates were compared using Standard Error of Difference (Sprague & Fogels 1976). Where a toxicant was not used, data were analysed in Minitab, using 1 and 2 way Analysis of Variance (ANOVA) to calculate significant differences ($p<0.05$) and *post-hoc* analysis using Tukeys Pairwise Analysis to locate the significant differences. Data can also be transferred to the graphics and data analysis package (Origin) to enable visual assessment of the data. Prior to use in Origin, data are manipulated in Microsoft Excel to derive averages, and 95 % confidence intervals.

Appendix I

BTT-I: Cladoceran immobilisation test – Stock culture maintenance

A.I.1 Primary cladoceran stock culture

Fleas are kept individually in small vials (45mL plastic vials with snap-on lids, lids have two air-holes) and are transferred to 30 mL aliquots of fresh water daily. The water fleas are kept in filtered Magela Creekwater and filtered Darwin tap water, fed FFV (Fermented Food with Vitamins) and algae and are kept in the incubators as is done during a test. 24–36 vials are kept at all times, but further vials may need to be maintained depending on experimental demands.

Every 3–4 days, neonates are collected to restart the stock (preferably from neonates produced in the second brood), to ensure continuation of species line. As for toxicity testing, neonates from the first brood are never used to restart the stock – the second brood is used preferentially over the third due to increased number of males present in the third brood.

A.I.2 Secondary cladoceran stock culture

A secondary stock of cladocera are maintained in filtered stream water and filtered Darwin tap water filled 1–2 L bowls in a separate location, as a precaution against contamination or accidents. This is a monospecific culture of cladocerans. Detailed notes are recorded in laboratory log books.

The secondary cladocera culture are fed every second day with algae and FFV (approximately 450 μL). When the second brood is produced by the primary cladocera stock culture, the excess water fleas are used to restart the secondary culture. The original water fleas are removed, the bowl cleaned and water renewed. The primary cladocera (and water) are added, along with approximately 50 cladocera from the secondary culture. Detailed notes are recorded in laboratory log books.

A.I.3 Sexual reproduction in cladoceran cultures

If the animals are stressed, which occurs if the culturing conditions are below optimum (eg. polluted water, little food, frozen algae fed to primary cultures etc) the adult diploid female may produce both diploid *male* and diploid *female* young.

In sexual reproduction mature diploid males mate with mature diploid females, and the females produce a different sort of egg, a resting egg. These special eggs are protected by thickenings of the brood chamber, which is called an ephippium. The ephippium and its eggs lie dormant (even after the adult female dies) until favourable conditions occur, allowing the eggs to hatch. The eggs have genes from both sexes, ie heterozygous, thus enabling the cladocera to better adapt to its changing environment, and ensure survival of the species. This phenomenon (the occurrence of a seasonal variation in the external appearance of specimens) is known as cyclomorphosis, and is a characteristic of many species of cladocera including *M. macleayi*.

It has not been determined if there is a difference in survival/behaviour etc between male and female cladocera in test conditions. However, based on the above information, it is assumed

that males may show a greater tolerance to adverse conditions, and hence may not react representatively to toxicants. This laboratory has not used males for testing purposes.

A.I.4 Recommended husbandry of cladocera

An incubator set to 27 ± 1°C houses a stock of asexually-reproducing (parthenogentic) water fleas kept in separate vials (usually 24–36 animals). These vials are 45 mL screw-capped vials with two 2 mm diameter ventilation holes per cap (Nylex type 12, Selby Scientific and Laboratory Equipment, are suitable). Four days prior to the test (eg on Thursday if the test starts on the following Monday) neonates from the second brood are collected for use as parents of the test neonates. The water and food is changed daily for each vial, and neat accurate records maintained on the culture health. Water is changed by transferring the water fleas (adults only if any brood present) using a pipette into clean vials. Each vial has 30 mL of fresh diluent water, with the water equilibriated to 27 ± 1°C prior to transfer of the water flea. FFV (Fermented Food with Vitamins) is added as a suspension at a rate of 30 µL/30 mL/day, and algal cells at a rate of 6×10^6 cells/30 mL/day. Neonates from the first brood are discarded. Neonates from the second brood are collected for the test animals (brood three neonates are avoided due to increased presence of males

A.I.5 Fermented Food with Vitamins (FFV)

Five grams of a commercially-available proprietary pelleted cichlid food (Wardley Products, New Jersey) and 0.25 g of dried and powdered alfalfa are homogenised in 250 mL of Milli-Q water for five minutes using a domestic food-blender. The mixture is then bubble-aerated for 2–3 days at ambient water temperature (25–34°C), after being covered with a fine mesh to ensure it does not become contaminated by flies, dust etc, and to allow gas exchange. At the end of this fermenting period (the mixture is determined to be pungent rather than putrid to smell), the mixture is collected into a beaker and placed at 4°C for 1 h and allowed to settle. The supernatant (approximately 150 mL) is then decanted carefully into another beaker and 0.3 g of calcium pantothenate and 1.5 mL of a stock Vitamin B_{12} solution (100 mg/L) is added to this beaker (Murphy 1970, Keating 1985). This is then divided into aliquots of about 1 mL in plastic vials, capped and frozen and clearly labelled with preparation date. An aliquot is thawed when needed, and resuspended by gentle shaking before use. The aliquot can then be refrigerated (4°C) but must be discarded one week after thawing.

A.I 6 Protocol for mass culture of algae

Two types of algal cultures are maintained:

(i) stock or ‘starter’ cultures

(ii) mass or ‘food’ cultures

The alga was isolated from Georgetown Billabong, and has been tentatively identified as a *Chlorella* sp. described by Ling (pers comm), and cultured as in the laboratory.

All steps outlined below should be carried out aseptically in a laminar flow cabinet using sterile media and glassware. Starter cultures are placed in the growth cabinet (approximately 40–50 µmol m^{-2} s^{-1}) set on a 12:12 light/dark cycle at 29 ± 1°C and are kept on a constant gentle-motion agitator plate. Culture flasks for mass culture of algae for food are placed in the growth cabinet (40–50 µmol m^{-2} s^{-1}), set on a 12:12 light/dark cycle at 27 ± 2°C and are continuously gently swirled. A mass culture should be prepared at least monthly.

A.I 6.1 Method for the preparation of algae using MBL

1. Approximately 5–6 days before commencing a mass culture, transfer ≈1 mL of algae from an older starter culture into a 250 mL Erlenmeyer flask to which 100 mL of MBL medium has been added. Cells in this new starter culture should be exponentially growing by the time they are used to inoculate the harvest food culture.
2. To prepare media for the algae culture, make up 4–6 L of MBL medium in 2 L Erlenmeyer flasks and adjust pH to between 7.1 to 7.3 using HCl acid. Stopper the flask with non-absorbent cotton wool bung and cover with aluminium foil to prevent contamination by airborne particles. Autoclave for 20 minutes (see table A.I.1 below for details on MBL preparation). Allow the media to sit at room temperature for at least 1 day before use to ensure adequate gas exchange.
3. Add 10 mL of starter culture to each 2 L flask of harvest media using a sterile pipette in the Laminar Flow Cabinet to inoculate the harvest culture. Place the flask in the growth cabinet, culture for 7–10 days (until harvest appears quite green) and then harvest the algae.

Table A.I.1 MBL medium solutions and procedure

	Ingredient	Stock Solution	Media Solution
1	Tris Buffer	100g/L	5mL/L
2	$NaNO_3$	85.24g/L	1mL/L
3	$CaCl_2.2H_2O$	36.76g/L	1mL/L
4	$MgSO_4.7\ H_2O$	36.97g/L	1mL/L
5	$NaHCO_3$	12.6g/L	1mL/L
6	K_2HPO_4	8.72g/L	1mL/L
7	Na_2EDTA	4.36g/L	1mL/L
8	$FeCl_3.6H_2O$	0.727g/L	1mL/L
9	Vitamins	*See below*	1mL/L
	Cyanocobalamin (Vitamin B12)		
	Thiamine hydrochloride (Vitamin B1)		
	d-Biotin (Vitamin H)		
10	**Trace metals**	In 1L add:	1mL/L
	$CoCl_2.6H_2O$	10mg/L	
	$CuSO_4.5H_2O$	9mg/L	
	$Na_2SiO_3.5H_2O$	7mg/L	
	$MnCl_2.4H_2O$	180mg/L	
	$ZnSO_4.7H_2O$	22mg/L	

Vitamin Stock

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

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

Note: These stocks can be stored frozen.

Preparation of Stock:

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

2. Add a few mL of Milli-Q water to dissolve.
3. Add 2.5 mL of the above biotin stock and 2.5 mL of the Vitamin B12 stock.
4. Make up to 250 mL with Milli-Q water. Use 1 mL of this stock per litre MBL media.

A.I.6.2 Procedure for making media

1. Add 1 ml of each stock solution per litre of Milli-Q water, except Tris Buffer–add 5 mL/L.

Note: These ingredients are stored at 4°C, and require replacing at 18–24 month intervals.

2. Adjust media to pH 7.1–7.3 using 25% HCl.
3. Pour MBL media into: 4–6 x 2 L flasks, such that there is 1 L per flask for the harvest culture.
4. Use a cotton bung to plug the top of the each flask. Cover the bung and mouth of the flask with aluminium foil. Record the date the media is autoclaved and media type on a strip of autoclave tape and place on alfoil.
5. Autoclave at 121^{o}C for 20 min.
6. Allow the media to cool to room temperature and leave overnight before inoculating.
7. Media may be stored at room temperature while not in use.

A.I.6.3 Protocol for harvesting algal cells

1. Aseptic techniques are not practical in the harvesting of algal cells but the work area should be as clean as possible. All dilution water (eg Magela Creek water) used in the following steps is sterilised by autoclaving.
2. 4 x 500 mL plastic tubes are filled with 400–500 mL of the algae culture. The tubes are placed in a refrigerated centrifuge fitted with a swing-out head. Tubes placed opposite each other are pair-wise balanced or matched to the nearest 0.1g, both for this step and all other steps. Any remaining algae culture can be spun in used tubes after the supernatant has been removed and the resuspended pellet transferred. The centrifuge is set to a rotor speed of 3500 rpm and a temperature of 15°C. A spinning time of 20 minutes is adequate.
3. The supernatant is removed through suction, using a glass Pasteur pipette directly attached to a venturi suction via flexible plastic tubing. Use of the venturi suction allows controlled removal of any bacteria growth that may become evident as a layer on the surface of the algal pellet. A sufficient quantity of water (~ 5mL) is left behind to allow resuspension of the pellet.
4. The resuspended pellets are combined and transferred into the minimum number of tubes possible – 60 mL tubes are used after the volume is significantly reduced. The tubes are spun at 2800 rpm for 20 minutes at 15°C.
5. The empty tubes are rinsed by adding 20–30 mL of sterile dilution water, capped and shaken to ensure total resuspension of pellet. To keep the volume of rinsing water used to a minimum, the same aliquot is transfered from one tube to another, and eventually to a final tube to be re-spun. This rinsing procedure is repeated twice, or until the final aliquot appears clear.
6. The above procedure is repeated until all algal cells are concentrated into one tube. These cells are then washed three times with sterile dilution water to remove any trace metals

(from the culture media) by spinning, removing the supernatant, adding approximately 50 mL sterile dilution water and shaking sufficiently to resuspend the pellet.

7. The pellet from step 6 is finally resuspended in approximately 50 mL of sterile dilution water.

8. The algal density of the suspension must be measured to calculate the required volume needed to be added to *Moinodaphnia macleayi* treatment vials to attain the pre-determined known cell density of 2×10^5 algae/mL. To measure algal density (see also Padovan 1992), transfer 1 mL of the suspension to a 100 mL volumetric flask and make up to the mark using sterile dilution water. Seal with Parafilm and mix well by inverting five times. Quickly break the seal and load the haemocytometer for a cell count. If the algal density is too high, try a greater dilution (eg add 0.5 mL concentrate to a 250 mL measuring cylinder). Count at least 100 cells in each of four chambers (re-seal and invert five times before loading the haemocytometer again), average the values and back–calculate to determine the algal density of the concentrate.

8. The algal suspension should be divided into 2mL aliquots and stored in the dark at 4°C. The algal feeding aliquot of 30×10^6 cells should always have a volume of less than 500 µL. If not, the algal concentrate should be re-centrifuged and the volume adjusted.

PROTOCOL SHEET

Project Name Djalkmara pre-release testing **Project Number:**

Test Number: 611I **Test Name:** 48h_Djalk_02 **Start Date:** 17/12/02

BTT: I-Cladoceran immobilisation test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	3000 mL	0 mL
B	0.3%		9 mL
C	1.0%		30 mL
D	3.2%		96 mL
E	10%		300 mL
F	32%		960 mL
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: 611I **Test Name:** 48h_Djalk_02

Toxicant: Djalkmara Billabong **Diluent:** Magela creekwater

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	As per lab manual	As per lab manual
Site	Djalkmara pumping station	Georgetown
Transportation	22 L jerry cans	22 L jerry cans
Comments	Collected by XX	Collected by XX

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: Chemical weighed out by:

Balance used: Stock solution prepared by:

TEST DETAILS			
BTT	I		
Species:	*Moinodaphnia macleayi*		
Start Date:	17/12/02		
Start Time:	10:00am		
Started by:	XX		
Incubator no:	Beige-1		
Temps OK?	Yes		
Test waters prepared by:	XX		
Supervisor:	XY		
Chem. analysis: **J/N,S/N & date** **Submitted by:**	NTEL – metals AGAL – TOC, alkalinity		
Departure from BTT / comments:			

FRESH water parameters

Test No: _611I	**Test:** 48h_Djalk_02	**Species:** *Moinodaphnia macleayi*	
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong	
PH:	**Cond:**	**pH:**	**Cond:**

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**					**Observer / Date:**
0	pH											
	Cond											
	DO											
1	pH											
	Cond											
	DO											
2	pH											
	Cond											
	DO											
3	pH											
	Cond											
	DO											
4	pH											
	Cond											
	DO											
5	pH											
	Cond											
	DO											

QC: pH stays w/I ±0.2 unit of Day 0 values for each Conc.; Cond for each test soln is w/I 10% of Day 0 values; DO conc. >70% air saturation value for each conc.

24 hour OLD water parameters

Test No: 611I	**Test:** 48h_Djalk_02	**Species:** *Moinodaphnia macleayi*
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**			**Observer/ Date:**
1	pH									
	Cond									
	DO									
2	pH									
	Cond									
	DO									
3	pH									
	Cond									
	DO									
4	pH									
	Cond									
	DO									
5	pH									
	Cond									
	DO									
6	pH									
	Cond									
	DO									

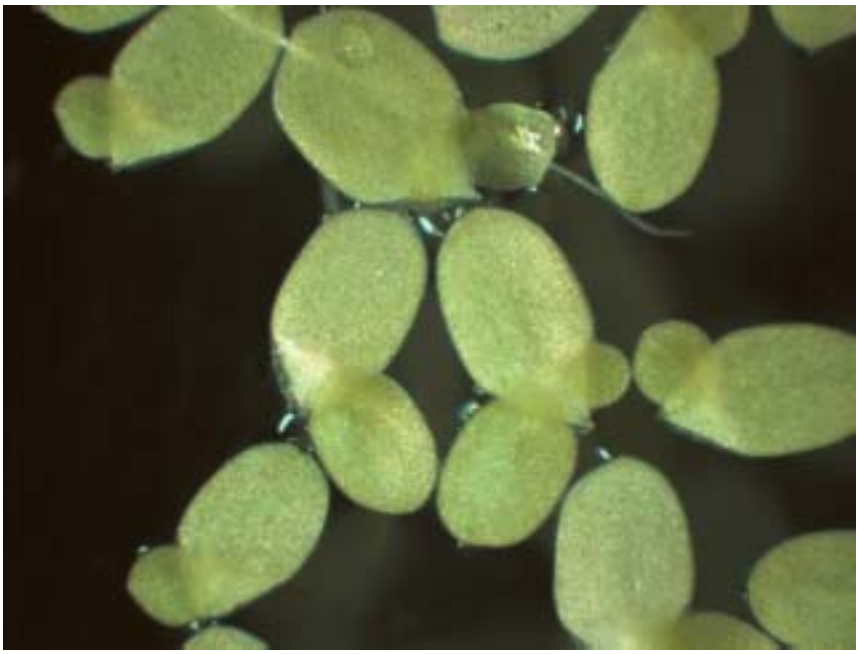
CLADOCERAN ACUTE SURVIVAL TEST

Test No.: 611I **Species:** *Moinodaphnia macleayi* **Concentration:**CONTROL

Test Name: 48h_djalk_02 **Start Date:** 17/12/02

Replicate/Observation		1	2	3	4	5	6	7	8	9	10	
Day 1	**No. org.**											**Observer/Date**
(24h)	**No. alive**											
	mortality:											**Checked by**
	Comments:											
Day 2	**No. org**											**Observer/Date**
(48h)	**No. alive**											
	mortality:											**Checked by**
	Comments:											
Sum of Juvenile Survival:												
Total No. animals:												

BTT-L: Lemna growth inhibition test



Lemna aequinoctialis Welwitsch

BTT-L: Lemna growth inhibition test

L.1 Objective

The objective of a test series (ie 3–4 definitive tests) is to determine the concentrations of a specified chemical or whole effluent that shows:

a) The No Observed Effect Concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the 10% Bounded Effect Concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk 1993);

b) The Lowest Observed Effect Concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared to the Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response becomes significantly ($P \leq 0.05$) lower than that of the control (Ahsanullah & Williams 1991);

c) The inhibition concentration concentration (IC_{50}), is the concentration of chemical in solution that is estimated to cause a 50% inhibition of a sublethal response of test organisms. This is measured as the 50% inhibition plant growth/frond numbers of test lemna (*Lemna aequinoctialis*) over 96 h (ASTM 1992).

L.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. The number of fronds are counted at the end of the test and from this the increase in biomass is calculated and compared to that of an appropriate control to determine the percentage inhibition of growth for each treatment.

L.3 Test organism

The test species is *Lemna aequinoctialis* Welwitsch (Lemnaceae, Spathiflorae), a small aquatic, flowering macrophyte commonly known as duckweed. The duckweeds are ecologically relevant test organisms in that they are primary producers and a source of food for water fowl, fish and small invertebrates. By floating in mats on the surface of still waters they provide habitat for a multitude of small organisms. Unlike many other of the evolutionary more complex plants their small size and fast growth rates make them ideal for testing in the laboratory.

Lemna selected for testing must be free of overt disease and gross morphological deformity (ie each plant has (i) fronds which are of uniform size and are a healthy green colour and (ii) a healthy root system). A ‘suitable’ test plant is one which has three fronds only – 2 mature fronds and one slightly smaller, less-developed frond. Test plants are obtained from stock cultures that are maintained in the laboratory as described in Appendix L.1. Original test lemna were collected from South Alligator River (Yellow Water Billabong) by C Camilleri, February 1997.

L.4 Dilution water

There are two dilution water types routinely used. The aim of the test will determine which water type is used.

L.4.1 25% CAAC

A 25% solution of the culturing growth medium, a variation of Hoagland's E (Cleland & Briggs 1969) and K (Maeng & Khudari 1973), was found to adequately maintain exponential growth of the plants for the duration of the test. This diluent is prepared by adding the specified quantities of stock solution to a volumetric flask that is partially filled with Milli-Q water. The pH of the water is adjusted to 6 ± 0.1 (Appendix L.2) and the flask is then filled to volume.

L.4.2 Natural stream water

Natural stream water is the receiving water taken upstream of the waste water discharge outlet (eg Magela Creek upstream of Georgetown pumping station latitude 12° 40' 28" longitude 132° 55' 52" – Wet season *or* Bowerbird billabong latitude 12° 46' 15", longitude 133° 02' 20" – Dry season). It should be collected in acid-washed plastic containers as close as is practicable to the start of the test, ie afternoon prior to test commencement, and stored at 4°C overnight. Within 48 hours of collection, the required amount of water for the test should be filtered through a fine pore size filter (eg Whatman No 42, 2.5 μm) capable of removing 'wild' zooplankton. The water should be stored in covered polyethylene containers at 4°C for a maximum period of three weeks.

L.5 Stock solutions

L.5.1 Chemical solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in an acid-washed, plastic container and refrigerated (4^oC). The source of the stock solution (eg date of preparation, by whom) is marked on the bottle and on an information sheet. Immediately prior to use for test water preparation the stock solution is allowed to equilibrate to room temperature.

L.5.2 Whole effluent solutions

Whole effluent stock solutions are collected from the designated site as close as is practicable to the start of the test, in an acid-washed plastic or glass container (depending on the chemical properties of the effluent). The sample should be kept sealed, clearly labelled and refrigerated at 4°C until required for test commencement.

L.6 Test solutions

Test solution preparation for lemna tests involves modification of diluent water in order to promote good lemna growth. To make modified stream water, concentrations of 144.32 mg/L KNO_3 (88.52 mg/L NO_3) and 23.36 mg/L KH_2PO_4 (18.4 mg/L PO_4) are required in the filtered stream water. Wagner and Hogan (2003) found this concentration of nitrate and phosphate to be the only nutrients required to maintain exponential growth over a 96 h period for the temperate freshwater *Lemna aequinoctialis*. A 500 mL batch of solution is prepared

for each treatment by adding the appropriate volume of stock solution to modified stream water in a volumetric flask. Aliquots (100 mL) of test solution are dispensed into each of three test containers and a sample bottle sent to an external laboratory for chemical analysis.

Appropriate volumes of test diluent (100, 99 or 98 mL) are added to labelled 250 mL Erlenmeyer flasks, stoppered loosely and autoclaved at 120°C for 20 min. These are allowed to cool overnight before the secondary stocks are added. The flasks are lined up in a laminar flow cabinet, their stoppers removed and placed behind them, then the appropriate volume of secondary stock is added using a 1000 µL dispensing pipette and sterile pipette tips. The rim of each flask is sterilised over a blue flame using a bunsen burner, stoppers are replaced and the flasks are gently swirled to mix the solution. Two or three 100 mL replicates per treatment are required (the number of replicates depends on the number of treatments in the test). An additional flask containing 200 mL is prepared for water parameter measurement and any chemical analyses required. The flasks are then ready to be inoculated with lemna.

L.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the lemna are placed, or the lemna themselves, should be chemically inert.

L.7.1 Container preparation

All containers (ie bottles,flasks etc) and equipment used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 µS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least three times with Millipore water (< 1 µS cm^{-1}); and
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with appropriate diluent or control water. Other equipment should be rinsed thoroughly with Millipore water (< 1 µS cm^{-1}) before use.

L.7.2 Temperature control

Tests are conducted at 29 ± 1°C using a constant temperature growth cabinet. During observation, test containers are removed from the growth cabinet for the minimum amount of time possible to avoid fluctuations in test solution temperature.

L.7.3 Photoperiod control

Tests are conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity ranges between 100–150 μmol m^{-2} s^{-1} (Photosynthetically Active Radiation) and is checked periodically using a light meter.

L.7.4 Equipment

- Constant temperature growth cabinet or growth room with light boxes and temperature recorder
- Light meter
- Laminar flow cabinet
- Bunsen burner and LPG supply and lighter
- 5 L polyethylene containers (to hold treatment solutions, one per solution)
- Refrigerator for storage of test and stock solutions
- Autoclave
- Autoclave tape
- 250 mL Erlenmeyer flasks, 3–4 per treatment (depending on no. of reps) – includes 1 flask for water quality parameters and chemistry
- Non-absorbent cotton-wool bungs
- Aluminium foil
- A-grade volumetric flasks
- 100 mL measuring cylinder with 1 mL graduations
- Calibrated 1 mL dispensing pipette and sterile tips
- Disposable sterilised inoculating loops
- Three sterilised 95 mL crystallising dishes (sterilise by wrapping in foil and autoclaving for 20 minutes at 120°C)
- Two acid-washed 95 mL crystallising dishes
- 100 mL disposable plastic vials with screw caps (for water parameter measurement)
- 70 mL acid-washed bottles (glass or plastic depending on toxicant) with plastic screw caps, for analytical chemistry
- Maximum-minimum thermometer
- Calibrated mercury thermometer
- pH meter, pH electrode and pH buffer solutions of 7.00 and 4.01
- Conductivity meter and electrode, calibrating solution
- Dissolved oxygen meter
- Random number generator
- Magnifying lamp (eg Maggy Lamp)
- Needle-nosed forceps

- 70% ethanol and Daylee towelettes for cleaning laminar flow cabinet
- Magnetic stirrer and stirrer bars

L.8 Test environment

The preparation and storage of test solutions, culturing of test plants, manipulation, and actual testing is carried out in premises free from harmful vapour, dust, and any undue disturbance. The preparation of test solutions and transfer of plants is undertaken in a laminar flow cabinet to maintain the axenic nature of the test solutions. All workers involved in any part of the test wash their hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure. Powder-free vinyl gloves are worn when working in the laminar flow cabinet.

L.9 Data recording

Test plants are observed for any fungal or bacterial contamination and test flasks randomised at 24 h intervals after the test commences (when t = 0 h). Plant numbers and water quality parameters are measured and recorded at the beginning and end of the test (t = 0 h and t = 96 h).

L.10 Test procedure

Day 0 (ie starting day of the test t = 0 h)

1. Prepare the test diluent, autoclave and allow to cool overnight.
2. Run UV light for 30 minutes, wipe down laminar flow cabinet with ethanol and light the bunsen burner.
3. Add secondary stock solutions to diluent and leave in the laminar flow cabinet.
4. In the laminar flow cabinet, divide the contents of two flasks of cultured lemna into three sterilised crystallising dishes.
5. Randomly line up labelled test flasks and remove bungs. Place bungs behind corresponding open flasks.
6. Systematically place one suitable plant into each flask using a new, sterile plastic inoculating loop for each plant.
7. Continue inoculating until each flask contains four plants (12 fronds), sterilising the rim of the flask by flaming when transfer is complete. Replace bungs.
8. Flasks that contain test water for water quality parameters and chemistry should be taken through the same procedure although they receive no lemna, then removed from the laminar flow cabinet for processing.
9. Check that each flask contains 4 x 3-fronded lemna.
10. Remove test flasks from cabinet and randomly place on one shelf of growth cabinet. Record the time as this is the start time for the test.
11. Decant approximately 60 mL of each treatment from the parameter/chemistry flasks into dry, acid-washed bottles; label bottles clearly then store, refrigerated, for chemical analyses. Acidify if needed.

12. With remaining water take dissolved oxygen, pH and conductivity measurements for all treatments.

Days 1–3

13 At the appropriate time ie at 24 hour intervals, remove all test flasks from growth cabinet.

14 Re-randomise their positions on the shelf and return to cabinet, recording any notable changes, or bacterial or fungal contamination.

Day 4 (ie 96 h end of test)

15 Remove flasks from growth cabinet then arrange, in order of ascending toxicity, on a clean bench.

16 Set up a Maggy Lamp or similar magnifying light source on the bench.

17 Make general observations of plant health eg size and colour of fronds, root size; and appearance of treatments eg cloudiness, precipitate formed, bacteria present.

18 Carefully pour the contents of the first treatment into a clean crystallising dish, ensuring that no lemna remain in the flask.

19 Using needle-nosed forceps gently remove each plant and count the fronds under magnification. A frond is counted, however small, when it is visible beyond the margin of the mother frond (Cleland & Briggs 1969). Place the frond on a tissue or white sheet of paper, discarding when count is complete.

20 Record the number of fronds on the appropriate information sheet.

21 Pour the test water, with fronds removed, back into the flask and set aside.

22 Rinse crystallising dish with Milli-Q water then wipe dry with lint-free tissue or Daylee towellette, or obtain a clean, dry dish.

23 Repeat procedure for the replicate/s of that treatment then combine the residual test waters and take subsamples for water quality parameters and analytical chemistry.

24 Repeat steps 17–23 for all flasks, always working in ascending concentration of toxicant.

25 Measure water quality parameters and process chemistry samples as for Day 0, steps 11 and 12.

Test is complete.

Calculation of percent inhibition of plant growth

The increase in biomass in all flasks is calculated by subtracting the intitial frond number from the final frond number.

In order to determine an IC50, the percent inhibition (% I) is calculated for each treatment using the following formula:

$$\% I = 100(M - X)/M$$

where:

M = average increase in biomass in the control test flasks,

X = increase in biomass in the treatment flasks.

L.11 Randomisation

Randomness is an important component of the experimental design. On each day a new set of random numbers must be used for the position of each flask in the incubator for the next 24 h period. The flasks are repositioned each morning after being swirled to aid gas exchange.

L.12 Avoiding bias

To avoid observer bias there should be at least two observers, however, this will not always be possble. Each observer randomly selects a replicate group to record each day, and observations commence with the next highest chemical concentration to that which was first observed the previous day. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section L.13, should be used.

L.13 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Magnesium (added as sulphate) is used in a concentration range from 10–50 mg/L. Synthetic water is used as diluent (appendix A.L.2). The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to magnesium. If the value falls outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

L.13 Acceptability of test data

The test data are considered acceptable if:

1. The recorded temperature of the growth cabinet remains within the prescribed limits (29 ± 1°C);
2. The frond number in any control flask at test conclusion is at least five times that at test start (ie 60 fronds/flask);
3. The recorded pH is within the prescribed limits;
4. The conductivity for each test solution is within ± 10% of the values obtained on Day 0.
5. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 29^{o}C;
6. The result of reference toxicity testing is within the set limits.

L.14 Analysis of test data

The endpoints of the lemna growth test are measured as the 96 h EC_{50} and the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC). The EC_{50} is the effective concentration giving 50% reduction in lemna growth over 96 h compared to the controls. This is calculated using Trimmed Spearman-Karber analysis (Hamilton et al 1977) or Maximum Likelihood Probit analysis ToxCalc™ (Toxcalc Version

5.0.23D, Tidepool Software, 1996). After testing the data for normality and homogeneity of variances, Dunnett's Multiple Comparison Test is used to determine which treatments are significantly different from one another. This information enables estimation of the LOEC and NOEC. Alternative statistical measures to the NOEC and LOEC can also be calculated. The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, is estimated using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, is estimated using the approach described by Ahsanullah and Williams (1991).

Appendix L

BTT-L: Lemna growth inhibition test – Stock culture maintenance

A.L.1 Primary lemna stock culture

Specimens of *L. aequinoctialis* were identified and collected from Yellow Water Billabong in February 1997 and brought to the ***eriss*** laboratory for culturing (Kym Brennan, pers comm). Initial stocks were grown in billabong water in small glass aquaria situated on a sunny aspect. Backup cultures of laboratory stock can be maintained in this way.

To obtain a population suitable for use in testing, ie an axenic isolate that could be cultured in a controlled stable environment, it was necessary to run growth trials.

250 mL Erlenmeyer flasks containing 100 mL of either billabong water or a synthetic growth medium (Appendix L.1), were autoclaved for 20 minutes at 120°C, before being inoculated with plants (10 fronds per flask) in a sterile environment (laminar flow cabinet) using sterile equipment and techniques. Both inocculants were placed in a constant temperature (29 ± 1°C) growth cabinet and observed for vegetative and bacterial growth. Bacterial growth was apparent in both treatments after several days but was worst in those flasks containing growth medium.

Over a period of three months, several concentrations of growth media were trialed and hyperchlorite treatments were used to obtain an axenic culture with good vegetative growth. The pH of the growth medium was also adjusted to 6 using a 10 g/L solution of KOH to better represent the pH of natural waters in the region. Although this adjustment caused a small amount of white precipitate to form, it did not appear to affect plant growth. A 50% growth medium with Milli-Q water as diluent was found to be most effective for maintaining a culture with weekly subculturing.

The hyperchlorite treatments varied in concentration and time of exposure. Ultimately the most severe treatment was needed to control bacterial growth, being a 5% solution in which the plants were immersed for 60 seconds. The cultures were observed daily for signs of bacterial infection, which was apparent as cloudy media and/or white slime on the roots of the plants. If there was evidence that bacteria was present, the following procedure was followed: in a laminar flow cabinet and using sterile inoculating loops, the plants were removed from the contaminated flask, dipped into a sterile container of deionised water to remove any visible bacteria, then transferred to a container of 5% hyperchlorite solution where they were gently agitated for 60 seconds. They were then placed in a clean flask of autoclaved media. After many weeks of persistent bacterial growth, the pre-wash with Milli-Q water during the hyperchlorite treatment proved to be the crucial extra step in eliminating its' recurrence. Whilst the plant growth and general health were retarded by the bleaching process, once the bacterial problem was removed and the treatments ceased, normal growth and health returned.

A.L.2 Culture Procedure

A.L.2.1 Growth medium

The growth medium used for culturing and testing lemna is a variation of Hoagland's E (Cleland & Briggs 1969) and K (Maeng & Khudari 1973). A 50% solution is used for

culturing and a 25% solution with sucrose omitted is used for testing, with the composition shown in table A.L.1 below.

Table A.L.1 Composition of the Growth Medium used in culturing and testing (NB ***eriss*** laboratory uses 50% of Hoagland's E and K for culturing and 25% for testing)

Ingredient	Stock solution (g/L)	Culture medium (mg/L)	Test diluent (mg/L)	Preparation
1. Sucrose	No stock	2050	Nil	Weigh and add directly to 1 L flask part filled with Milli-Q water before other nutrients are added. Do not add to test diluent.
2. KH_2PO_4	50.32	25.16	12.08	Add 5 mL/L for culture and 2.5 mL/L for test.
3. KNO_3	88.88	44.44	22.22	Add 5 mL/L for culture and 2.5 mL/L for test.
4. $Ca(NO_3)_2.4H_2O$	94.40	47.20	23.60	Add 5 mL/L for culture and 2.5 mL/L for test.
5. $MgSO_4.7H_2O$	50.00	25.00	12.50	Add 5 mL/L for culture and 2.5 mL/L for test.
6. Na_2EDTA	9.0	4.5	2.25	Add 0.5 mL/L for culture and 0.25 mL for test.
7. Tartaric acid	3.0	1.5	0.75	Add 0.5 mL/L for culture and 0.25 mL for test.
8. Micronutrients				Add 0.5 mL/L for culture and 0.25 mL for test.
H_3BO_3	2.86	1.43	0.715	
$ZnSO_4.7H_2O$	0.22	0.11	0.065	
$Na_2MoO_4.2H_2O$	0.12	0.06	0.030	
$CuSO_4.5H_2O$	0.08	0.04	0.020	
$MnCl_2.4H_2O$	3.62	1.81	0.905	
$FeCl_3.6H_2O$	5.40	2.70	1.35	

This solution is made up routinely every four weeks as follows:

1. Half fill a clean 1 litre volumetric flask with Milli-Q water.
2. Add stock solutions as described in table, using appropriate dispensing pipettes and clean tips.
3. Fill to volume with Milli-Q water.
4. Place flask on a magnetic stirring plate, add a clean stirrer and check pH (should be around 4.6).
5. Turn on stirring plate then add small volumes of KOH (10g/L solution) to the flask, checking pH after each addition until it reaches and remains at 6 ± 0.1.
6. Dispense 100mL of this solution into each of 10 clean acid-washed 250 mL Erlenmeyer flasks.
7. Loosely stopper the flasks with bungs (non-absorbent cotton wool covered with Daylee towelette and fastened with autoclave tape) then cover with aluminium foil squares that are large enough to extend past the neck of the flask and firmly press the foil to fit.

8. Place a strip of autoclave tape over the top of the foil-covered bung and clearly write the date on the tape with a permanent marker.
9. Place the flasks in an autoclave and set it to run for 20 minutes at 120°C.
10. When flasks have sufficiently cooled, store them in a clean, cool place until required. Allow them to stand at least overnight before use to ensure temperature and gas equilibration.

Note: Discard medium after four weeks from preparation date and/or if there are any signs of bacteria or fungus present.

A.L.2.2 Inoculation

Cultures of lemna are subcultured weekly, with older cultures being retained for several weeks as back-up in case of contamination. Restarting procedure is as follows:

1. Wipe the laminar flow cabinet down with an ethanol wipe.
2. Wipe equipment required with ethanol wipe and place in the laminar flow cabinet. ie two flasks of CAAC solution, two disposable loops (one as a back up), gloves, lighter and bunsen burner.
3. Turn on the UV light in the laminar flow cabinet for 30 minutes before starting procedure.
4. Turn on LP gas supply, turn off UV light, open cabinet and turn on air flow and light.
5. Place one flask of the previous weeks lemna culture flasks in the cabinet.
6. Put on gloves, remove the foil and place them behind their respective flasks.
7. Remove the bung, and flame the lip of the flask over the bunsen burner. This is done by holding the flask at an angle so the rim is exposed to the flame and passing the lip over the flame so the whole of the rim is sterilised. The blue portion of the flame is used for sterilisation.
8. Use the opened disposable inoculating loop to carefully remove 5 – 7 plants from the culture flask.
9. Flame the lip of the flask and replace the bung.
10. Flame the lip of the new flask and place the lemna in the growth medium.
11. Repeat this process for the other flask of growth medium
12. Turn off the bunsen burner, put the foil back on the flasks and remove all equipment from the laminar flow cabinet.
13. Wipe out the cabinet with an ethanol wipe, turn off the gas, and turn off the air flow and light.
14. Label the newly inoculated flasks with the date of inoculation, and note on the older flask that it has been opened.
15. Enter the details in a log book, along with observations of plant health etc.
16. Replace flasks in growth cabinet.

NB: always perform a visual check for contamination of the old lemna cultures prior to restarting.

PROTOCOL SHEET

Project Name Djalkmara pre-release testing **Project Number:**

Test Number: 611L **Test Name:** Lemna_Djalk_02 **Start Date:** 17/12/02

BTT: L-Lemna growth test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	3000 mL	0 mL
B	0.3%		9 mL
C	1.0%		30 mL
D	3.2%		96 mL
E	10%		300 mL
F	32%		960 mL
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: 611L **Test Name:** Lemna_Djalk_02

Toxicant: Djalkmara Billabong **Diluent:** Magela creekwater

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	As per lab manual	As per lab manual
Site	Djalkmara puming station	Georgetown
Transportation	22 L jerry cans	22 L jerry cans
Comments	Collected by XX	Collected by XX

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: Chemical weighed out by:

Balance used: Stock solution prepared by:

TEST DETAILS			
BTT	L		
Species:	*Lemna aequinoctialis*		
Start Date:	17/12/02		
Start Time:	10:00am		
Started by:	XX		
Incubator no:	Beige-1		
Temps OK?	Yes		
Test waters prepared by:	XX		
Supervisor:	XY		
Chem. analysis: **J/N,S/N & date** **Submitted by:**	NTEL - metals AGAL – TOC, alkalinity		
Departure from BTT / comments:			

FRESH water parameters

Test No: 611L	**Test:** Lemna_Djalk_02	**Species:** *Lemna aequinoctialis*	
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong	
PH:	**Cond:**	**pH:**	**Cond:**

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**					**Observer / Date:**
0	pH											
	Cond											
	DO											
1	pH											
	Cond											
	DO											
2	pH											
	Cond											
	DO											
3	pH											
	Cond											
	DO											
4	pH											
	Cond											
	DO											
5	pH											
	Cond											
	DO											

QC: pH stays w/I ±0.2 unit of Day 0 values for each Conc.; Cond for each test soln is w/I 10% of Day 0 values; DO conc. >70% air saturation value for each conc.

24 hour OLD water parameters

Test No: 611L	**Test:** Lemna_Djalk_02	**Species:** *Lemna aequinoctialis*
Diluent: Magela creekwater		**Toxicant:** Djalkmara Billabong

Conc. / Day		**A Control**	**B 0.3%**	**C 1.0%**	**D 3.2%**	**E 10%**	**F 32%**			**Observer/ Date:**
1	pH									
	Cond									
	DO									
2	pH									
	Cond									
	DO									
3	pH									
	Cond									
	DO									
4	pH									
	Cond									
	DO									
5	pH									
	Cond									
	DO									
6	pH									
	Cond									
	DO									

LEMNA POPULATION GROWTH

Test No.: 611L **Species:** *Lemna aequinoctialis*

Test Name: **Start Date:** **Initial no./dish:** **Replicate no.:** 1

Day	Concentration / Observation	A	B	C	D	E	F	G	H	
1	No. of Plants									Date:
	No. of Fronds									
	General Appearance									Observer:
	Comments									Checked by:
2	Fungal Growth?									Date:
	Bacterial Growth?									
	General Appearance									Observer:
	Comments									Checked by:
3	Fungal Growth?									Date:
	Bacterial Growth?									
	General Appearance									Observer:
	Comments									Checked by:

LEMNA POPULATION GROWTH (continued)

Test No.: 611L **Species:** *Lemna aequinoctialis*

Test Name: **Start Date:** **Initial no./dish:** **Replicate no.:** 1

Day	Concentration / Observation	A	B	C	D	E	F	G	H	
4	**No. of Plants**									**Date:**
	No. of Fronds									**Observer:**
	Plant Health									
	Treatment Appearance									
	Increase in Biomass									
	Percent Inhibition									
	Comments									**Checked by:**

BTT-M: Hornwort growth inhibition test

Hornwort *Ceratophyllum demersum* L, showing whorls individually and on plant

BTT-M: Hornwort growth inhibition test

M.1 Objective

The objective of the test is to determine the concentration of a specified chemical or whole effluent that shows:

a) The No Observed Effect Concentration (NOEC), which is the highest concentration at which no statistical difference ($P > 0.05$) is found between exposed and unexposed (or control) specimens. This can be compared with the 10% Bounded Effect Concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra & Van Ewijk 1993);

b) The Minimum Detectable Effect Concentration (MDEC), which is defined as the concentration at which the response became significantly ($P < 0.05$) lower than that of control (Ahsanullah & Williams 1991);

c) The median effect concentration (EC_{50}), is the effective concentration to cause a 50% reduction in biomass (growth) or total length over 96 h of test hornworts (*Ceratophyllum demersum*).

M.2 Principle of the test

A standard size of healthy *Ceratophyllum demersum* plants are exposed to a range of concentrations of a toxicant for 96 h under controlled conditions. The weight and length of the plant are measured at the beginning and end of the test, representing an increase in biomass which is compared to an appropriate control to determine the percentage inhibition of growth for each treatment.

M.3 Test organism

The test species *Cerotophyllum demersum* L (Magnoliophyta; Ceratophyllaceae) was sampled from the South Alligator River (12°52'S, 132°30'E) in the Northern Territory of Australia by S Jacobs. *C. demersum* is a free-floating, submerged, rootless, perennial flowering macrophyte that reproduces both asexually (primarily via vegetative fragmentation) and sexually (Parsons & Cuthbertson 2001). The plant provides an important ecological role in aquatic ecosystems by providing habitat and a food source for aquatic biota including fish and invertebrates.

C. demersum selected for the tests must be free of deformity and of similar dimension. The size of the plant should be five whorls in length (cut with scissors) including a growing tip and free of a secondary growing bud. Test plants are obtained from stock cultures, which are maintained at test conditions for at least three weeks.

M.4 Dilution water

The dilution water is a synthetic water that maintains exponential growth over 96 h (table M.1). The water is prepared by adding specific quantities of inorganic stock solutions to Milli-Q water of a known volume and aerated over night to produce a homogeneous solution.

Table M.1 Nominal inorganic composition of synthetic water[A]

Physico-chemical parameter	Total concentration for hornwort synthetic water
pH	7.0 ± 0.2
Temperature (°C)	27.0 ± 1.0
Na	25 mg L^{-1}
K	1.9 mg L^{-1}
Ca	5.6 mg L^{-1}
Mg	5.9 mg L^{-1}
Cl	44 mg L^{-1}
SO_4	9.3 mg L^{-1}
HCO_3	19 mg L^{-1}
NO_3	450 µg L^{-1}
PO_4	46 µg L^{-1}
Fe	300 µg L^{-1}
Al	79 µg L^{-1}
Mn	52 µg L^{-1}
Zn	0.88 µg L^{-1}
Cu	0.81 µg L^{-1}
Pb	0.11 µg L^{-1}
Cd	0.045 µg L^{-1}

A Dissolved organic carbon values were below their typical analytical detection limit (0.20 mg L^{-1})

M.5 Stock solutions

All stock solutions were prepared in Milli-Q water using analytical grade reagents and stored at 4°C in clean, inert, labelled containers. These are stored for a maximum of three months before being replaced to reduce possible contamination and ensure consistent concentrations in the solutions.

M.6 Test solutions

A sufficient volume (150 L) of the test solution should be made for the experiment. The stock solutions are allowed to reach room temperature (27°C) and are added to the volume of test water in the correct amounts. This now forms the test media which is then aerated, pH adjusted (7.0 ± 0.2), covered and left over night to equilibrate. Equal volumes of this solution are dispensed among eight 60 L polyethylene containers. The toxicant, of varying amounts, is added to seven of these containers leaving one for the control. All containers are labelled with the nominal concentration of toxicant that is used for the test.

M.7 Apparatus and test equipment

All materials used in the test, that come into contact with the plant specimen and solutions, should be clean to reduce test solution contamination and variability.

M.7.1 Container preparation

All containers (ie containers, bottles) used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999 Micro) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Millipore water (< 1 µS cm^{-1}) for the rinse cycle;
- Allow to air dry.

OR

- Immerse in a 1–3% detergent solution (eg Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 10% HNO_3 solution for 24 h;
- Thoroughly rinse at least three times with Milli-Q water (< 1 µS cm^{-1});
- Allow to air dry.

Note: Immediately before use, the test containers are rinsed with the appropriate test water concentration. All other equipment is rinsed thoroughly with Milli-Q water (< 1 µS cm^{-1}) before use.

M.7.2 Temperature control

Tests are conducted at 27 ± 1ºC using a climatically controlled room and checked periodically with a maximum-minimum thermometer.

M.7.3 Photoperiod control

Tests are conducted with a 12 h light:12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity ranges between 100–150 µmol m^{-2} s^{-1} and is checked periodically using a light meter.

M.7.4 Equipment

- Constant temperature growth room with temperature recorder
- Light meter
- Milli Q water purification system or equivalent
- A-grade glass volumetric flasks
- Analytical grade reagents
- 60 L polyethylene containers – to hold treatment solutions, one per concentration
- 1 L polyethylene containers for the stock solutions
- Refrigerator for storage of test and stock solutions
- 2 L polyethylene containers, three per treatment
- 13 mm diameter polyethylene mesh (gutter guard) – enough for dividers in test containers
- Aeration – via the use of air stones under low pressure
- Calibrated 1 mL and 5 mL dispensing pipette and sterile tips

- Disposable sterilised inoculating loops
- 60 mL acid-washed bottles (glass or plastic depending on toxicant) with plastic screw caps, for analytical chemistry
- Maximum-minimum thermometer
- Calibrated mercury thermometer
- pH meter, pH electrode and pH buffer solutions of 4.01, 7.00 and 10.00
- 0.05 M HCl and 0.05 M NaOH for pH adjustment
- Ruler or caliper with mm gradings
- Analytical balance (0.0000 g) and weight boats
- Paper towel
- Refrigerator for storage of test and stock solutions
- Data sheets

M.8 Test environment

The preparation and storage of test solutions, culturing of test *C. demersum*, manipulation, and actual testing is carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test wash their hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

M.9 Data recording

The test plants are observed at 24 h intervals for a change in appearance. The pH of the test solutions is measured at the same time and adjusted accordingly. Plant weight and length are recorded at the beginning and the end of the test (t = 0 and t = 96 h).

M.10 Test procedure

Day 0 (ie starting day of the test t = 0 h)

1. Prepare the background synthetic water (up to 500 L; table A.M.1) and allow to equilibrate overnight.
2. Partition the synthetic water into 60 L containers, add the toxicant and mix well (shake container). Allow at least 2 h to equilibrate.
3. Cut one and a half times as many plants that are needed, to the appropriate size (using mm ruler or calipers, starting length approximately 20 mm, weight 20–25 mg) with stainless steel scissors.
4. Determine wet blotting error (See Appendix A.M.2).
5. Rinse test containers (2 L) in the corresponding water and fill to 1.9 L (10 mm from the top of the container).
6. Insert acid-washed plastic mesh to divide the container into four sections (three sections for each replicate plant and one section for an airstone).

7. Place in the air stone under low pressure.
8. Randomly select cut plants, weigh and measure until there is enough for the test. Plants are randomly allocated to the various test containers.
9. Rinse plants in the corresponding concentration and add to the appropriate section of the container, using a plastic inoculating loop.
10. Repeat step 8 until all containers have three plants and an airstone.
11. Randomly place the containers under the light. Record the time and this is the start time for the test.
12. Immediately take equal subsamples of solution for chemical analysis from both the treatment containers (15 mL from each) into acid-washed 60 mL labelled containers; acidify (pH < 2) with nitric acid and refrigerate.
13. Measure pH and adjust if necessary.

Day 1 (24 h):

14. Measure pH and adjust if necessary.

Day 2 (48 h):

15. Take water samples in the same manner as step 12.
16. Renew test solutions completely by careful decantation.
17. Measure pH and adjust if necessary.

Day 3 (72 h):

18. Measure pH and adjust if necessary.

Day 4 (96 h):

19. Take water samples in the same manner as step 12.
20. Remove containers from the light then arrange, in order of ascending toxicity, on a clean bench.
21. Measure the total length and blotted weight of the plant and repeat until all containers have been measured, always working in ascending concentration of the toxicant.

Test is complete

M.11 Calculation of percent inhibition of plant growth

The increase in weight (biomass) and length for each concentration is calculated by subtracting the initial weight or length from the final measurement

In order to determine an EC_{50}, the percent inhibition (% I) is calculated for each treatment using the following formula:

$$\% I = 100(M - X)/M$$

where: M = average increase in biomass/length in the control test flasks, and

X = increase in biomass/length in the treatment flasks.

M.12 Randomisation

Randomness is an important component of the experimental design. On each day a new set of random numbers must be used for the position of each container in the incubator for the next 24 h period.

M.13 Avoiding bias

To avoid observer bias there should be at least two observers, however, this will not always be possible. Each observer randomly selects a replicate group to record each day, and observations commence with the next highest chemical concentration to that which was first observed the previous day. Occasional checks should be made on the incubator performance (ie constant temperature, light intensity, and their variation) by placing replicates in different incubators. If significant differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section M.15, should be used.

M.14 Reference toxicants

The use of reference toxicants enables the response of the test organism to be assessed over time to ensure the response is reproducible. This process also checks the proficiency of operators and laboratory standards. Copper (added as chloride) is used in a concentration range from 0.5–30 ug/L. Synthetic water is used as diluent (appendix A.M.3). The EC_{50} value, calculated from the concentration-response curve, should fall between 3SDs of the mean on the quality control chart for the test species exposed to copper. If the value falls outside 2SD of the mean it is a warning that there may be something wrong with the test (note that one in twenty samples will fall outside 2SDs just by chance).

M.15 Acceptability of test data

The test data are considered acceptable if:

1. The recorded temperature of the test solutions remains within the prescribed limits (27 ± 1°C);
2. There is a 25% increase in control biomass over 96 h;
3. There is < 20% variability in control growth;
4. The recorded pH is within the prescribed limits;
5. The result of reference toxicity testing is within the set limits.

M.16 Analysis of test data

The endpoints of the hornwort growth test are measured as the 96 h EC_{50} and the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC).

The EC_{50} is the effective concentration giving 50% reduction in growth over 96 h compared to the controls. This is calculated using either (i) non-linear regression analysis (Origin Version 6.0, Microcal Software, 2001) or (ii) Trimmed Spearman-Karber analysis (Hamilton et al 1977) or (iii) Maximum Likelihood Probit analysis ToxCalc™ (Toxcalc Version 5.0.23D, Tidepool Software, 1996). After testing the data for normality and homogeneity of variances, Dunnett's Multiple Comparison Test is used to determine which treatments are significantly

different from one another. This information enables estimation of the LOEC and NOEC. Alternative statistical measures to the NOEC and LOEC can also be calculated.

The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, is estimated using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, is estimated using the approach described by Ahsanullah and Williams (1991).

Appendix M

BTT-M: Hornwort growth inhibition test — Stock culture maintenance

A.M.1 Primary hornwort stock culture

Ceratophyllum demersum were cultured in a climate-controlled room, in 400 L polypropylene aquaria containing dechlorinated tap water (table A.M.1) on a 12:12 h light/dark cycle (Philips 400 W sodium halide lighting, 120 µmol m^{-2} s^{-1}) at 27 ± 1°C. The water was part-replaced (50% by volume) weekly to maintain chemical composition. Florapride™ (Tetra, Blacksburg USA) a nitrate- and phosphate-free aquatic plant food, was also added weekly (60 mL) to assist plant growth rates. To maintain stock numbers, plants were fragmented (by hand or with scissors) on a weekly basis, maintaining 5–10 leaf whorls per plant. Plants were selected at random for use in toxicity tests.

Table A.M.1 Chemical composition of dechlorinated tapwater[A]

Parameter	Dechlorinated Tap Water
Conductivity	76 (7.2) µS cm^{-1}
DOC	4800 (520) µg L^{-1}
Na	9800 (910) µg L^{-1}
K	810 (69) µg L^{-1}
Ca	2300 (350) µg L^{-1}
Mg	2500 (360) µg L^{-1}
Fe	460 (91) µg L^{-1}
Al	120 (15) µg L^{-1}
Mn	39 (6.2) µg L^{-1}
SO_4	4000 (910) µg L^{-1}
Cl	16000 (870) µg L^{-1}
HCO_3	11000 (1300) µg L^{-1}
NO_3	70 (10) µg L^{-1}
PO_4	35 (7.3) µg L^{-1}
Zn	1.0 (0.12) µg L^{-1}
Cu	1.3 (0.14) µg L^{-1}
Co	0.28 (0.051) µg L^{-1}
Ni	0.35 (0.057) µg L^{-1}
Pb	0.21 (0.077) µg L^{-1}
Cd	0.059 (0.038) µg L^{-1}

[A] Geometric mean total concentrations (and 95% CL) over 12 months (n = 48)

A.M.2 Determination of wet blotting error

Three plants were randomly chosen from the culture aquarium and cut into five whorls. Plants were blotted on paper towel and weighed individually and recorded and then placed back into the water while the others were weighed. This process was repeated 20 times for each plant,

and the mean and standard deviation were determined. From this, a mean co-efficient of variation of 1.7% was calculated. This indicates minimal variation in the method used for wet blotting the plant, and provides confidence when determining differences in biomass between toxicant exposures.

A.M.3 Preparation of synthetic water

Prepare synthetic water as follows:

Table A.M.3 Inorganic components of synthetic water

Salt[A]	Chemical Addition[B]	Concentration (µg L^{-1})
$MgSO_4 \cdot 7H_2O$	255 g L^{-1}	22000 Mg^{2+}
	Add 20 mL per drum	8900 SO_4^{2-}
$MgCl_2 \cdot 6H_2O$	138 g L^{-1}	3600 Mg^{2+}
	Add 50 mL per drum	11000 Cl^-
$CaCl_2 \cdot 2H_2O$	230 g L^{-1}	5600 Ca^{2+}
	Add 20 mL per drum	9900 Cl^-
NaCl	103 g L^{-1}	18000 Na^+
	Add 100 mL per drum	28000 Cl^-
$NaHCO_3$	58.6 g L^{-1}	7100 Na^+
	Add 100 mL per drum	19000 HCO_3^-
KCl	81.3 g L^{-1}	1900 K^+
	Add 10 mL per drum	1700 Cl^-
$FeCl_3 \cdot 6H_2O$	31.9 g L^{-1}	300 Fe^{3+}
	Add 10 mL per drum	560 Cl^-
$Al_2(SO_4)_3 \cdot 18H_2O$	22.0 g L^{-1}	79 Al^{3+}
	Add 10 mL per drum	420 SO_4^{2-}
$MnCl_2 \cdot 4H_2O$	8.43 g L^{-1}	52 Mn^{2+}
	Add 5 mL per drum	67 Cl^-
$ZnSO_4 \cdot 7H_2O$	0.174 g L^{-1}	0.88 Zn^{2+}
	Add 5 mL per drum	1.5 SO_4^{2-}
$CuCl_2 \cdot 2H_2O$	0.0978 g L^{-1}	0.81 Cu^{2+}
	Add 5 mL per drum	0.90 Cl^-

A Minor impurities in the listed salts precluded the need to add Cd, Pb, NO_3 and PO_4 to achieve the nominal concentrations.

B Each drum contains 225 L

PROTOCOL SHEET

Project Name Hornwort Cu and water hardness testing **Project Number:**

Test Number: 611B **Test Name:** hornwort_Cu_02 **Start Date:** 17/12/02

BTT: M-hornwort growth test

Details:

	TREATMENT	DILUENT	TOXICANT
A	Control	150 L	0 L
B	8ppb		
C	11.75ppb		
D	12.75ppb		
E	21.25ppb		
F	35.5ppb		
G			
H			
I			
J			

Quality Control:

Chemistry:

A 60 mL sample of each treatment was taken, acidified with 1% nitric acid and stored at 4°C until analysed.

Other:.

TOXICITY TEST DETAILS

Test Number: **Test Name:** Hornwort_Cu_02

Toxicant: Cu **Diluent:** Synthetic water

Water Collection/Preparation Details		
	TOXICANT	**DILUENT**
Date	16/12/02	16/12/02
Time	am	am
Method	pump	Pump
Site	Boat ramp	Boat ramp
Transportation	25 L Polyethylene barrels	25 L Polyethylene barrels
Comments	Collected by XX	Collected by XX

OR

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: 17/12/02 Chemical weighed out by:

Balance used: Sartorius Stock solution prepared by:

TEST DETAILS			
BTT	M		
Species:	*Ceratophyllum demersum*		
Start Date:	17/12/02		
Start Time:	11am		
Started by:	XX		
Incubator no:	Growth room		
Temps OK?	yes		
Test waters prepared by:	XX		
Supervisor:	XY		
Chem. analysis: **J/N,S/N & date** **Submitted by:**			
Departure from BTT / comments:			

Old water parameters

Test No: 611M	**Test:** Hornwort_Cu_02	**Species:** *Ceratophyllum demersum*	
Diluent: Synthetic water		**Toxicant:** Cu	
pH: 6.95	**Cond:** NR	**pH:** NR	**Cond:** NR

Conc. / Day		**A Control**	**B 8 ppb Cu**	**C 11.75 ppb Cu**	**D 12.75 ppb Cu**	**E 21.25 ppb Cu**	**F 35.5 ppb Cu**					**Observer / Date:**
1	pH											
	Cond											
	DO											
2	pH											
	Cond											
	DO											
3	pH											
	Cond											
	DO											
4	pH											
	Cond											
	DO											

QC: pH stays w/i ±0.2 unit of Day1 values for each Conc.; Cond for each test soln is w/I 10% of Day 1 values; DO conc. >70% air saturation value for each conc.

HORNWORT POPULATION GROWTH

Test No.: 611M **Species:** *Ceratophyllum demersum*

Test Name: Hornwort_Cu_02 **Start Date:** 17/12/02 **Initial no./dish:** 3 **Replicate no.:** 1

Measured Cu Concentration	Day 0 weight g	Day 4 weight g	Percentage Increase	Average % Increase	% of population growth relative to control
Control					
8 ppb					
11.75 ppb					

HORNWORT POPULATION GROWTH (continued)

Test No.: 611M **Species:** *Ceratophyllum demersum*

Test Name: Hornwort_Cu_02 **Start Date:** 17/12/02 **Initial no./dish:** 3 **Replicate no.:** 1

Measured Cu Concentration	Day 0 weight g	Day 4 weight g	Percentage Increase	Average % Increase	% of population growth relative to control
12.75					
21.25					
35.5					

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