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BTT-S 96 hour gastropod egg production test

Figure 1 *Amerianna cumingi*

1 Objective

The objective of this test is to determine the concentration of a particular test chemical/sample that affects the reproductive output (ie egg production) of the pulmonate gastropod *Amerianna cumingi* (Fig 1). This information can then be used alongside data from other aquatic organisms (from different taxonomic groups and trophic levels) to estimate the risks associated with releasing that particular chemical/sample to the freshwater environment.

2 Principle of the test

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

3 Test organism

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

Snails used in testing are obtained from flow-through tank cultures maintained in filtered Darwin tap water at 28 ± 2 °C in the ***eriss*** ecotoxicology aquaculture laboratory. Snails are fed the outer green leaves of iceberg lettuce after rinsing in deionised water and sprinkles of fish flake (Sera san, Heinsberg, Germany) daily. Broodstock are collected from the Jabiru Field Station (JFS) approximately every six months (or as required) and the offspring produced from these snails are used in testing. As such, the snails used in tests have been maintained under controlled laboratory conditions their entire life time. Previous work undertaken using snails cultured in the outdoor tubs at the JFS resulted in much greater variability (control percent confidence limits of 41 and 47) than what is routinely obtained using laboratory cultured snails (control percent confidence limits of < 25%).

Snails selected for testing must be between 10-13 mm in length from the tip of the anterior end of the shell running down the middle of the shell to the shoulder of the posterior end (do not include the spire when measuring length; Fig 2). Snails of adequate size must also be free of overt disease or scaling and damage of the shell. Snails should not be used in testing if the body cavity is not firmly attached to the shell and appears to be pulling back from the edge of the shell, as this may suggest that the snail is not of optimal health. When collecting test snails they should be carefully removed from their substrate (tank glass or lettuce) so as not to damage them. Applying a small amount of pressure to the posterior end of the muscular foot will encourage the snail to release its grip on a substrate with minimal stress on the individual. Caution should be taken to ensure that flatworms (if present in the culture tank) are not accidentally transferred along with the snails into the test containers. Any egg masses that may be present on the shells of snails must also be gently wiped off before the snail can be used in testing.

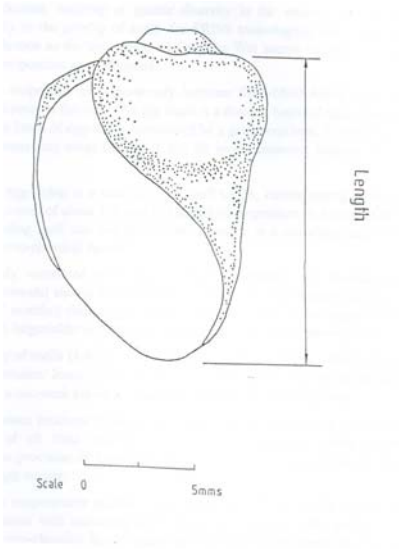

Figure 2 Ventral view of the shell of *A. cumingi* showing the points from which length is measured (Jones 1992)

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.

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 scenario for assessing toxicity, as under these conditions many toxicants (particularly metals) are more bio-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 (listed in Table 1) to Milli-Q water (< 1 µS cm^{-1}) in acid-washed polyethylene containers, as close as practical to the start of the test (see appendix A.E.6 for directions). 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 at 4°C until use.

Table 1 Mean nominal composition of the synthetic water

Physico-chemical parameter	Total concentration
pH	6.0 ± 0.15
Temperature (°C)	27 ± 1
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}

4.2 Natural stream water

Natural Magela Creek water is collected upstream of any influence of the Ranger Mine waste water discharge outlet. In the wet season water is collected upstream of the Georgetown Pumping Station (latitude 12° 40' 28'', longitude 132° 55' 52'') and from Bowerbird Billabong (latitude 12° 46' 15'', longitude 133° 02' 20'') during the dry.

This water should be collected in clean acid-washed gerry cans as close as possible to the start date of the test and stored at ≤4°C. The gerry cans should be removed from the refrigerator the afternoon before the test begins and left to warm to the room temperature of the Aquaculture lab (approx 28°C). Due to the large volumes of creek water that are required for this test, the water is not filtered prior to use.

5 Stock solutions

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 at 4°C. The container should be labelled with the date of preparation and initials of the person responsible. The container of test solution should be allowed to equilibrate to room temperature prior to use for test water preparation.

5.2 Whole effluent samples

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

6 Test solutions

22 L of unfiltered Magela Creek water is required for each treatment. Test solutions are prepared in bulk at the start of a test and stored in 22 L gerry cans in the cool room (4°C) until required. Test solutions are made up in four 5 L volumetric flasks and one 2 L volumetric flask by adding appropriate volumes of stock solution to Magela Creek water and then pouring the test solution into a gerry can for refrigeration. Each day 5 L of each treatment water is poured from the gerry cans into 5 L plastic (HDPE) bottles which are placed in the incubator overnight to warm for the following day's water renewals.

7 Water chemistry sampling and analysis

At the commencement of a test, water from each treatment is sampled in order to determine whether the correct concentration of the test chemical has been prepared or to understand the key constituents of a sample. The sample is taken from an additional test replicate that has undergone the exact preparation process as the solution used to expose the snails.

The analytes to be measured are selected according to the aim of the toxicity test and may include major ions, trace metals, nutrients, dissolved organic carbon and alkalinity. The decision to analyse for total concentrations or just the dissolved fraction of each of these analytes also depends on the objective of the experiment.

Measurements of a standard suite of metals are also made on the control waters to enable detection of common laboratory contaminants or metals that may modify toxicity (eg. Ca) and/or are common in natural waters. This standard suite consists of Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb, SO_4, Se, U and Zn.

Information on the methods used to sample, preserve and store samples are described below for each analyte group.

Major ions and trace metals

Samples to be analysed for soluble metals are filtered through a 0.45 μm cellulose nitrate filter (Millipore) using a 50 mL Terumo syringe (Elkton) directly into a 70 mL high density polyethylene (HDPE) sample bottle (Azlon). Prior to filtering the water samples, the syringes and filters should be rinsed twice with Milli-Q water. The filtered sample is acidified to

approximately 0.7% HNO_3 by adding 10 µL of 69% Aristar HNO_3 (BDH) for every mL of sample (determined by weighing sample bottles before and after sample addition).

Samples for total metal analysis are dispensed into 70 mL HDPE sample bottles and acidified to approximately 3.5% HNO_3 by adding 50 µL of 69% Aristar HNO_3 for every mL of sample.

Both the soluble and total metal samples are stored at 4°C until being submitted to the Northern Territory Environmental Laboratories (NTEL, Berrimah, Northern Territory, Australia) for analysis. Samples should be submitted within two weeks of the test being completed.

Nutrients

Samples to be analysed for soluble nutrients (NO_2, NO_3, NH_3, PO_4) and chlorine are filtered through a 0.45 µm PVDF filter (Millex-HV, Millipore) using a 50 mL Terumo syringe. Syringes and filters are rinsed as described above. Sample bottles are squeezed to remove air, then capped and frozen, and sent for analysis at NTEL as soon as possible after sampling. Samples for total N and P analyses are dispensed directly into 70 mL HDPE bottles and frozen with no air space.

Dissolved organic carbon and alkalinity

Samples should be poured into 500 mL Azlon plastic bottles (rinsed once in the sample) and refrigerated until being sent via overnight courier to the Australian Water Quality Centre in Bolivar, SA, for analysis. Note that the samples should be sent as soon a practicable after sampling to allow the analyses to be undertaken within a fortnight of collection. Extreme care must be taken in packing the samples to avoid breakage and they should be transported within a polystyrene esky with an ice brick so that they remain cool.

Ammonia

At the end of each 24 hour period, old water samples are collected by pooling water from each of the three replicates for each treatment. Ammonium (NH_4^+) concentrations of these samples are measured immediately using an Aquamerck test kit.

For a test to be valid, ammonium concentrations for all 24 hour old samples must be ≤1.0 mg/L. Observations of the snails throughout testing suggest that at an ammonium concentration of 1.0 mg/L, control snail pairs still produce egg numbers that are within the acceptable range of 50–250 eggs (based on the last ten years of in-situ monitoring of a control site upstream of Ranger mine). This test limit of 1 mg/L ammonium is conservative according to the trigger values provided in the Water Quality Guidelines; 2.57 mg/L total ammonia nitrogen at pH 6 and 2.18 mg/L at pH range 7 (ANZECC & ARMCANZ 2000).

Over the last six years the natural pH of unfiltered water collected from Bowerbird and Georgetown Pumping Station of Magela Creek has been between 5.6–7.1. At pH <7.0 and 30°C only 0.8% of total ammonia nitrogen exists as toxic un-ionised ammonia (ANZECC & ARMCANZ 2000). At pH 6 just 0.26% of total ammonia exists in the form of the toxic ammonia (NH_3) (ANZECC & ARMCANZ 2000). Thus while the total ammonia limit of 1.0 mg/L should be met for a test to be acceptable, it is likely that the majority of testing would be conducted around pH 6.0, where ammonia would mostly be present in the form of non-toxic NH_4^+.

8 Apparatus and test equipment

8.1 Container preparation

All containers, flasks and equipment (other than 22 L gerry cans) are prepared in the following way:

- Acid washed in 5% nitric acid bath for 24 hours
- Detergent washed in a dishwasher (Gallay Laboratory 999 Micro) using Gallay Clean A phosphate free detergent and Elix Reverse Osmosis (Elix) water.

The inner surface of 2 L test beakers that are used to hold the test solutions are also silanised with Coatasil and heated to 60°C overnight, prior to acid washing and dishwashing. This silanising prevents the loss of metal from solution due to adsorption to the test containers by inhibiting the adsorption of metals to the glass surface.

Gerry cans are washed by rinsing five times with Elix water and partially filling with 5% nitric acid. The gerry can should be laid down on its side and rotated periodically over two days to ensure that all internal surfaces have been soaked in the acid. The acid should then be tipped out and the gerry can rinsed again at least five times with Elix water. The pH of water from the final rinse should be checked with a pH strip and compared to the pH of Elix water to ensure that all acid has been removed.

8.2 Equipment

- Light-tight constant temperature incubator
- Milli-Q water purification system
- Refrigerator (set at 4°C)
- pH, electrical conductivity and dissolved oxygen meters
- A-grade volumetric flasks (2 L and 5 L)
- Chemicals and reagents
- Analytical balance and weigh boats
- 70 – 200 mL high density polyethylene and/or glass sample bottles
- Automatic adjustable pipettes (100µL, 1 mL and 5 mL) and measuring cylinders (100 mL)
- 5 L polyethylene containers and 22 L gerry cans
- 2 L glass beakers (silanised); 3 beakers per treatment
- Perspex tubes to house snail pairs (diameter 500 mm, length 700 mm)
- Squares of 0.5 mm mesh fabric (approximately 700 mm x 700 mm)
- Clips cut from PVC pipe (outer diameter 550 mm) into sections approximately 7–8 mm wide. Each section will require a small gap (approximately 20 mm) to be cut out of the ring so that the clips can be squeezed to fit into the inside of the perspex tube. These clips are then used to hold the mesh over both ends of each perspex tube.
- Aquarium heater (200 W)
- Aquarium air pump and vinyl airline tubing

- Long Pasteur pipettes
- Nally bin filled with Magela Creek water at 30 ±1°C
- Vernier calipers
- Watchglasses
- Dissecting microscope with bright field/dark field illumination
- Random number generator
- Fresh, crisp outer leaves of iceberg lettuce
- Lettuce cutter; 9–10cm length of PVC tubing with inner diameter 200 mm which has one end sharpened
- Disposable gloves, apron and lab coat
- Colorimetric ammonium test kit (eg Aquamerck, Merck, Darmstadt, Germany)

8.3 Incubators

Tests are conducted at 30 ± 1°C using a constant temperature incubator. During observation and water exchange the test containers are removed from the incubator for the shortest time possible to avoid temperature fluctuation. Fresh water for daily renewals is warmed to 30 ± 1°C overnight in the incubator prior to use. Tests are conducted with a 12 h light:12 h dark photoperiod. The light intensity of the incubators range from 30–100 $\mu molm^{-2}s^{-1}$.

9 Area for test preparation

The preparation of test solutions should be carried out in an area with ample space and which is free of contamination from harmful vapours, dust or disturbance. Throughout the test, workers should also take care not to introduce any contaminants during daily observations and water exchanges by washing hands and arms and wearing disposable gloves. When testing substances hazardous to human health it is recommended that staff wear disposable aprons on top of lab coats to prevent spillage of toxicants through lab coats and onto the skin.

10 Recording data

Test snails are observed and data recorded at 24 hour intervals until completion of the test at 96 hours. Observations made at the end of the first 24 hour period are designated as Day 1 observations, while observations at the end of the second 24 hour period as Day 2 observations etc.

11 Test procedure

Test preparation the day prior to the starting date

1 Water chemistry sample bottles should be labelled and weighed (if the samples will require acidification). The label should include the test code, the treatment, date, the percentage acid the sample contains and some way of identifying where the sample has come from ie '***eriss***'. Each bottle must be weighed before and after the sample has been added to calculate the sample volume. Once the sample volume is known, the volume of acid required to acidify each sample can be calculated.

2 Ensure there are sufficient clean silanised 2 L beakers for the test and label them with the treatment and replicate number.

3 Snail tubes are prepared by closing up one end of the tube with mesh and clip and placing these on a large perspex tray to carry over to the snail tank. This tray is then covered with another to avoid contamination of the vials with dust.

4 A gerry can of water will be required for each treatment. These should be removed from the refrigerator and left overnight in the Aquaculture lab (L.06) prior to a test to allow time to warm to 28 ± 2 °C.

5 Place a 40 L Nally Bin on a trolley (preferably at waist height) and fill the container with unfiltered Magela Creek water. Cover the container to prevent evaporation and contamination, and allow the water to warm overnight in the Aquaculture lab (L.06). An aerator can be set up but the water will not require aeration until Day 0.

6 Ensure that there is sufficient fresh outer lettuce leaves for the test.

7 Prepare a test folder that contains the appropriate data sheets for the test.

Day 0 (start date of test)

1 Prepare the test solutions as outlined in Section 6. For each treatment, dispense 1.75 L into each of three 2 L beakers, cover with glad wrap and equilibrate to 30 ± 1°C in an incubator. Fill 5 L bottles with the test solutions for each treatment for Day 1 and leave them in the incubator until the next day.

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

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

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

5 Isolate the test snails from the culture tank, measuring each snail to ensure it is between 10–13mm in length. As snails are picked from the tank, a pair should be carefully placed in each tube along with two discs of lettuce and the tube sealed off with a piece of mesh and PVC clip. (This is done as quickly as possible out of water.) Tubes are permanently labelled with treatment and replicate number and are selected randomly to avoid allocating differences in the snails' health or size to certain treatments. Note: for large-scale tests, this procedure works best with two people; one person to measure and place the snails in the tubes and another person to add the lettuce, seal the tubes with mesh and clip and place them in the acclimation tub.

6 Once all tubes contain a pair of snails, the trolley with the acclimation tub can be moved alongside the bench where the snails will be added to the treatments.

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

8 Snail tubes are taken from the acclimation tub and placed in the appropriate replicate (according to the label on the tube) until each beaker contains 6 snail tubes.

9 The beakers are re-covered with glad wrap and then randomly positioned in the incubator using a random number sheet.

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

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

Day 1

1 Cut lettuce discs and keep refrigerated until required.

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

3 Pour approximately 30 mL of waste water from each of the replicates into a polycarbonate vial to measure dissolved oxygen, conductivity and pH (these can be measured at a later stage). Use 5 mL of this pooled sample to measure the ammonium concentration (NH_4^+) with a colorimetric ammonia test kit. This should be done immediately to ensure accurate analysis.

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

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

6 All six tubes are then removed from each beaker, the old water is discarded and each beaker is filled with approximately 1.75 L of new water before the snail tubes are placed back in the beaker. The beaker is not rinsed prior to refilling. Approximately 60 mL of this new water needs to be set aside in a polycarbonate 100mL vial for measuring the parameters dissolved oxygen, pH and conductivity.

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

8 This procedure is repeated for all the remaining treatments working up the concentration gradient.

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

Days 2–3

1 Steps 1–9 of Day 1 are repeated on Day 2. Steps 1–8 are repeated on Day 3

Day 4

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

1 Remove Treatment A replicates from the incubator. Uncover beakers and handle one tube at a time, removing mesh and clips from both ends. Remove the snails (with care to prevent damaging any egg masses) and discard them into a waste container with water. Count and record the number of egg masses on the tubes and stack the tubes in a nally bin of water. (to prevent the egg masses from drying out).

2 Repeat this for the six tubes in each of the replicates for Treatment A.

3 A sample of water from each replicate should be kept for measuring dissolved oxygen, conductivity, pH and ammonium as described for Day 1.

4 Repeat the above steps for the remaining treatments.

5 The test snails are euthanased by placing the waste container in a freezer.

6 Each tube is then examined under a dissecting microscope (10 x magnification) and the number of eggs in each egg mass is counted and recorded for each pair of snails.

12 Randomisation and avoiding bias

On each day a new set of random numbers must be used to assign the position of each beaker in the incubator. Randomness is an important part of the experimental design. 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. The beakers will need to be taken out of their random order for daily water renewal and feeding. Following this they must be positioned in the incubator for the next 24 hours following a new set of random numbers.

13 Acceptability of test data

The dataset of the test is considered acceptable if:

1 The recorded temperature of the incubator remains within the prescribed limits

2 80% or more of the test control snails are alive

The data of each test group is considered acceptable if:

1 Egg numbers of the control are between 30–260 eggs per pair of snails (this range is based on control data from tests with *A.cumingi* throughout 2004–2006. See Figure 3).

2 The dissolved oxygen concentration is greater than 70% throughout the test at 30 ± 1°C

3 The conductivity for each test solution (new water) is within 10% of the values obtained on Day 0

4 The ammonium concentrations for each test solution are ≤1.0 mg/L (as explained in Section 7).

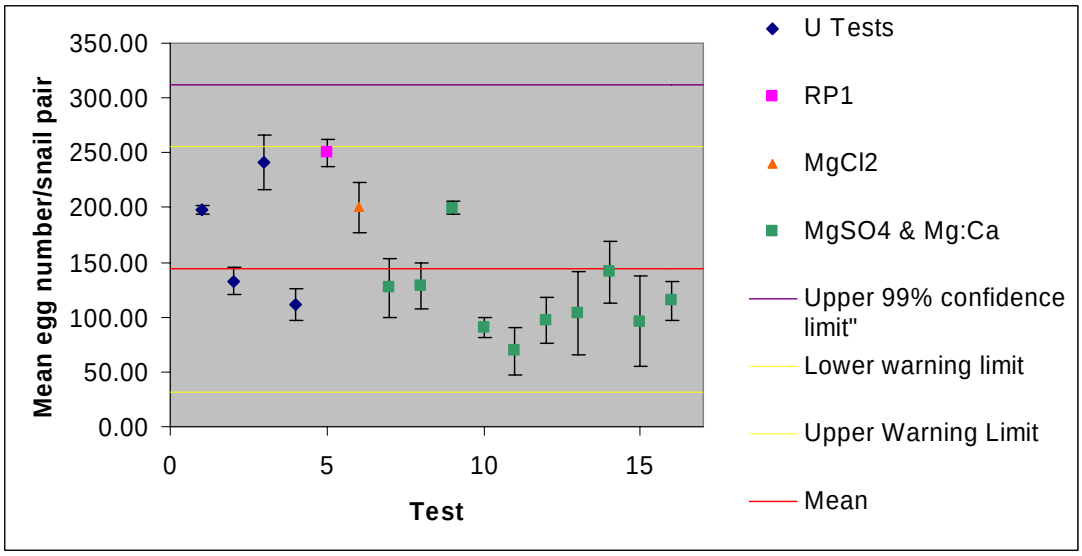

Figure 3. Control data from 16 snail tests conducted between 2004–2006 showing mean number of eggs per snail pair over 96 h and the minimum acceptable egg number based on 2 standard deviations from the mean

14 Analysis of test data

14.1 Summarising the raw data

Raw data (ie the number of eggs in each egg mass laid by each pair of snails) should be entered into an excel spreadsheet to calculate the total number of eggs produced by each pair and the mean number of eggs per pair in each replicate group (ie six pairs). The mean, standard deviation and % co-efficient of variation (%CV) for each treatment are calculated from the three replicate means. The %CV of the control treatment is used as an indicator of the within treatment variability for the experiment and the statistical analyses are undertaken on the mean number of eggs per pair for each replicate.

14.2 Analysing the summarised data

Background

Earlier test protocols prepared by the ***eriss*** ecotoxicology laboratory recommend the use of hypothesis testing approaches (eg Dunnett's test) to calculate the no effect concentration (NOEC; highest test concentration that is not statistically different from the control) and lowest effective concentration (LOEC; lowest test concentration that is statistically different from the control) as measures of toxicity. This approach has been used widely by ecotoxicologists, with NOEC's in particular, being utilised in deriving water quality guidelines for contaminants (ANZECC & ARMCANZ, 2000). Hypothesis testing has, however, been shown to have numerous limitations, such as dependence on the concentrations tested and sensitivity to within-treatment variability (de Bruijn & Hof, 1997).

Since the early 1990's, researchers have been discussing the potential use of point estimation techniques, where different effects concentrations (eg. IC_{10}, IC_{50}) are estimated by interpolating from a model equation that is fitted to the overall concentration response, as an alternative to hypothesis testing (Chapman et al 1996; Warne, 1998). The advantages of this method are that because the effect concentrations are interpolated, they are less dependent on the test concentrations used and the precision of this value can be quantified using confidence intervals (de Bruijn & Hof, 1997). In addition, because a model is fitted to the entire concentration response relationship, information such as the range of sensitivity of the organism (ie the slope of the distribution) can be compared across tests/species/contaminants. That the sensitivity of the method is not dependent on the within-treatment variance is of particular relevance to this protocol because egg production by *A. cumingi* is highly variable across individuals (Fig 3). Several considerations, such as ensuring that the observed concentration-response relationship is strong and that at least one treatment gives a partial effect, are important for the successful use of point estimation techniques (Moore & Caux, 1997), however, good experimental design can ensure that these are met.

For the reasons listed above, the ***eriss*** ecotoxicology laboratory is in the process of adopting point estimation/linear regression techniques for deriving toxicity estimates. It must be acknowledged that at this point in time, there has been insufficient discussion amongst the ecotoxicology community with regard to choosing the most appropriate experimental design and the criteria for model selection. As such, it is envisaged that the procedure described below will be modified as further understanding of the approach and consensus amongst ecotoxicologists is achieved.

For a single toxicity test

Where only one toxicity test is undertaken, for example as a once-off toxicity assessment of a whole effluent, the data should be analysed using the Linear Interpolation method in ToxCalc V.5.0.23F (Tidepool Scientific Software). The point estimates selected for reporting would most commonly be the IC_{10} and IC_{50}, although ultimately the selection of appropriate point estimates will be dependent on the overall objective of the test. In general, the IC_{10} is considered to represent a concentration that is unlikely to cause an ecological effect, while the IC_{50} is a robust measure of toxicity (ie. confidence intervals are generally at their minimum around the midpoint of a distribution).

Where tests are repeated

In cases where a toxicity test is repeated several times, for example to confirm the toxicity of a particular contaminant in Magela Creek water, an analysis of covariance should be undertaken in Statistica V.7 on normalised data (ie presented as a % of the control) to compare the concentration response (ie slope and y-intercept) of the different tests. Where the response is found to be similar, the data can be pooled so that point estimates can be interpolated from one model equation that is fitted to all the data. An appropriate model is selected in Minitab Release 14.13 from the sigmoidal family of distributions by comparing several models and selecting the one that provides the best fit (ie highest R^2) and uses the lowest number of parameters.

Where a significant difference between the responses of repeated tests has been found, an assessment of why the tests were not repeatable should be made and, provided the quality of all tests is assured, they should be analysed individually using linear interpolation. The geometric mean of the point estimates should then be taken to give an overall measure of toxicity.

Appendix – Test sheets

Protocol sheet

Project Name **Project Number:**

Test Number: 808S **Test Name:** Amer_U_01 **Start Date:** 5/03/07

BTT: S

Details:.

	TREATMENT (NOMINALS)	TREATMENT (MEASUREDS)	DILUENT
A	Control		See calc^{n} sheet
B	10 µg/L		
C	20 µg/L		"
D	40 µg/L		"
E	80 µg/L		
F	160 µg/L		
G			
H			
I			
J			

Quality Control:

Chemistry:

Other:

Toxicity test details

Test Number: 808S **Test Name:** Amer_U_01

Toxicant: U **Diluent:** MCW

Water Collection/Preparation Details		
	TOXICANT	**DILUENT (Control water)**
Date	Made from stock – see below	Coll: 1/3/07 Filt: 2/3/07
Time		
Method		As per lab manual
Site		
Transportation		
Comments		

IF TOXICANT IS PREPARED FROM STOCK SOLUTION:

Date of Preparation: 01/03/06

2°Stock solution prepared by:

1°stock made up by:

TEST DETAILS			
BTT	S		
Species:	Amerianna cumingi		
Start Date:	5/3/07		
Start Time:			
Started by:			
Incubator no:			
Temps OK?			
Test waters prepared by:			
Supervisor:			
Chem. analysis: **Submitted by:**	NTEL – metals		
Departure from normal protocol-comments:			

Fresh water parameters

Test No: 808S	Test: Amer_U-01	Species: *Amerianna cumingi* Diluent: MCW Toxicant: U

Treatment		A	B	C	D	E	F	G	H	I		Initial/Date:
Day 0 NEW water	pH											
	Cond											
	DO_2											
	Temp											
1 NEW water	pH											
	Cond											
	DO_2											
	Temp											
2 NEW water	pH											
	Cond											
	DO_2											
	Temp											
3 NEW water	pH											
	Cond											
	DO_2											
	Temp											
4 NEW water	pH											
	Cond											
	DO_2											
	Temp											

QC: pH stays w/I ±0.1 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; temp is that displayed on the pH meter at the time of measuring pH

24 hour old water

Test No: 808S		Test: Amer_U-01		Species: *Amerianna cumingi*		Diluent: MCW	Toxicant: U			
Treatment		A	B	C	D	E	F	G	H	Initial//Date:
Day 1	pH									
OLD water	Cond									
	DO_2									
	Temp									
2	pH									
OLD	Cond									
water	DO_2									
	Temp									
3	pH									
OLD	Cond									
water	DO_2									
	Temp									
4	pH									
OLD	Cond									
water	DO_2									
	Temp									

Snail reproduction test

AMMONIA CONCENTRATIONS (mg/L NH4+)

Test no.: **800S** **Test name:** **Amer_U_01** **Species:** **Amerianna cumingi**

	Day							
Treatment	1	2	3	4	5	6	7	8
A								
B								
C								
D								
E								
F								
G								
H								
Observer initials								

Snail reproduction test

DAILY EGG MASS COUNTS

Test no.: 800S **Test name:** Amer_U_01 **Species:** *Amerianna cumingi* **Observer initials:** **Day:** 1

Replicate	Pair	A	B	C	D	E	F	G	H
1	1								
	2								
	3								
	4								
	5								
	6								
2	1								
	2								
	3								
	4								
	5								
	6								
3	1								
	2								
	3								
	4								
	5								
	6								

Snail reproduction test

FINAL DAY EMBRYO COUNTS

Test no.:800S Test name: Amer_U_01 Species: *Amerianna cumingi* Observer initials: Day: 4 Treatment: A

Replicate	Pair	No. embryos	Total no. embryos	Comments
1	1			
	2			
	3			
	4			
	5			
	6			
2	1			
	2			
	3			
	4			
	5			
	6			
3	1			
	2			
	3			
	4			
	5			
	6			

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