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Environmental monitoring protocols to assess potential impacts from Ranger minesite on aquatic ecosystems: In situ toxicity monitoring – freshwater snail, *Amerianna cumingi*, reproduction test

Supervising Scientist Division

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GPO Box 461, Darwin NT 0801

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Executive summary

The Supervising Scientist Division (SSD) operates an integrated chemical (including radiological), physical and biological monitoring program to ensure protection of the aquatic ecosystems of the ARR from the operation of uranium mines in the region. This stream monitoring program is an independent assurance program, unlike the compliance and check water chemistry monitoring programs of the mining company (Ranger) and the NT government regulator respectively.

The techniques and 'indicators' used in the monitoring program satisfy two important needs of environmental protection: (i) the early detection of significant changes in measured indicators to avoid short or longer term ecologically important impacts; and (ii) assessing ecological or ecosystem-level effects by way of measured changes to surrogate indicators of biodiversity.

SSD has prepared protocols for the measurement programs required to implement each of these monitoring techniques. For each technique, two types of protocols have been prepared, high-level protocols and detailed operational manuals. This document is the high-level protocol, describing the science underpinning one of the biological early detection techniques, namely use of freshwater snails for in situ toxicity monitoring.

This protocol for the snail reproduction toxicity monitoring technique provides an overview of the monitoring principles and objectives, experimental and statistical design, test, data analysis and impact assessment procedures and reporting requirements.

Preamble and acknowledgments

Full descriptions of the field procedures, methods for data collation and worked examples of data analysis required to implement the methods described in this protocol are contained in a companion 'Operational manual'. The manual is the working document used by monitoring staff at the Jabiru Field Station to conduct the snail toxicity monitoring test. The Operational manual is in a loose-leaf ring-bound form, allowing for ready revision and update. Revisions must be approved by the SSD Monitoring Support Unit (Darwin) before the Operational manual can be updated.

The specific material contained in the Operational manual includes:

- datasheet pro forma for experimental and QA/QC results,
- details of all field and laboratory procedures used for toxicity monitoring over the four-day test period,
- details of all statistical procedures used for data analysis,
- animal husbandry requirements and procedures,
- summary information on key references and supporting studies.

Acknowledgements

This document builds upon the original methods protocol developed by Helen Allison (of ***eriss***) in the mid 1980s (Allison et al 1989). Subsequently, a number of ***eriss*** personnel and students have been involved in the further development of the original methods contained in the protocol, and the development of new ones: Chris Humphrey, Brendan Lewis, Duncan Buckle, Ian Brown, Abbie Spiers, James Boyden, Jane Suggit, Kelly Jones, Robert Luxon, Bob Pidgeon, Mark Ziembicki and Christy Davies.

Many volunteers, including local indigenous people, have assisted with the animal husbandry and fieldwork over this time.

The advice received from, and past collaboration with, staff from the former ERA field laboratory at Jabiru East is gratefully acknowledged. In particular, in a period of proposed technology transfer, ERA provided data for some tests reported in 1996, 1998, 1999, 2000 and 2001 (Supervising Scientist 2002, p 20).

Dr Keith McGuinness from Charles Darwin University has provided valuable advice during recent years on the use of statistical procedures for data analysis. ***eriss*** Director Dr David Jones provided critical review of the draft protocol.

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Environmental monitoring protocols to assess potential impacts from Ranger minesite on aquatic ecosystems: In situ toxicity monitoring – freshwater snail, *Amerianna cumingi*, reproduction test

Supervising Scientist Division

1 Introduction

1.1 Objective

Detection under field experimental conditions, of any[1] effects of dispersed mine waste-waters upon the egg production capacity of freshwater snails.

1.2 Background

Toxicity monitoring is a form of biological monitoring that provides for detection of adverse effects arising from developments such as mining, by evaluating lethal and sub-lethal responses of captive organisms exposed to effluent waters. The collection of data on sub-lethal effects, as precursors to possible adverse effects at the population level, may provide early detection of such effects and assist in determining their ecological importance. The current test organism deployed for (in situ) toxicity monitoring is the freshwater snail, *Amerianna cumingi*. This species has two key virtues in relation to its suitability as a test organism for toxicity monitoring in the local receiving waters. Firstly, it (has been shown to be amongst the most sensitive of a group of six local freshwater species to both uranium and magnesium (important constituents in wastewaters from the Ranger Uranium Mine, NT), as assessed by laboratory toxicity testing (Hogan et al 2010, van Dam et al in 2010, respectively). Secondly, it is also relatively easy to culture under controlled indoor and outdoor conditions.

The present method of test deployment uses an in situ technique, in which snails held in floating containers in the creek are exposed to a continuous supply of creek waters passing through the containers. This technique is relatively low maintenance, is portable, and provides for excellent water flow-through and contact conditions for the test organisms.

To date, toxicity monitoring has been conducted in Magela Creek upstream of the mine (control site) and at an 'exposed' site located approximately 400 m downstream of the Ranger mine's water quality compliance point at gauging station GS8210009. These are the sites at which the SSD also conducts its continuous and grab sampling water quality monitoring programs (Brazier et al 2010). The two sites are approximately 6 kilometres apart (Figure 1). Toxicity monitoring was extended, on a pilot basis, to Gulungul Creek, during the 2009–10

[1] Enhanced (hormetic) effects may indicate the response of snails to low-level contaminant concentrations in receiving waters which may be sufficient cause to trigger management action.

wet season. The catchment of this creek is at increasing risk of impact by mine-related activities. While testing in Gulungul Creek continued during the 2010–11 wet season, details of the testing and data analysis procedures for this creek are not reported in this protocol.

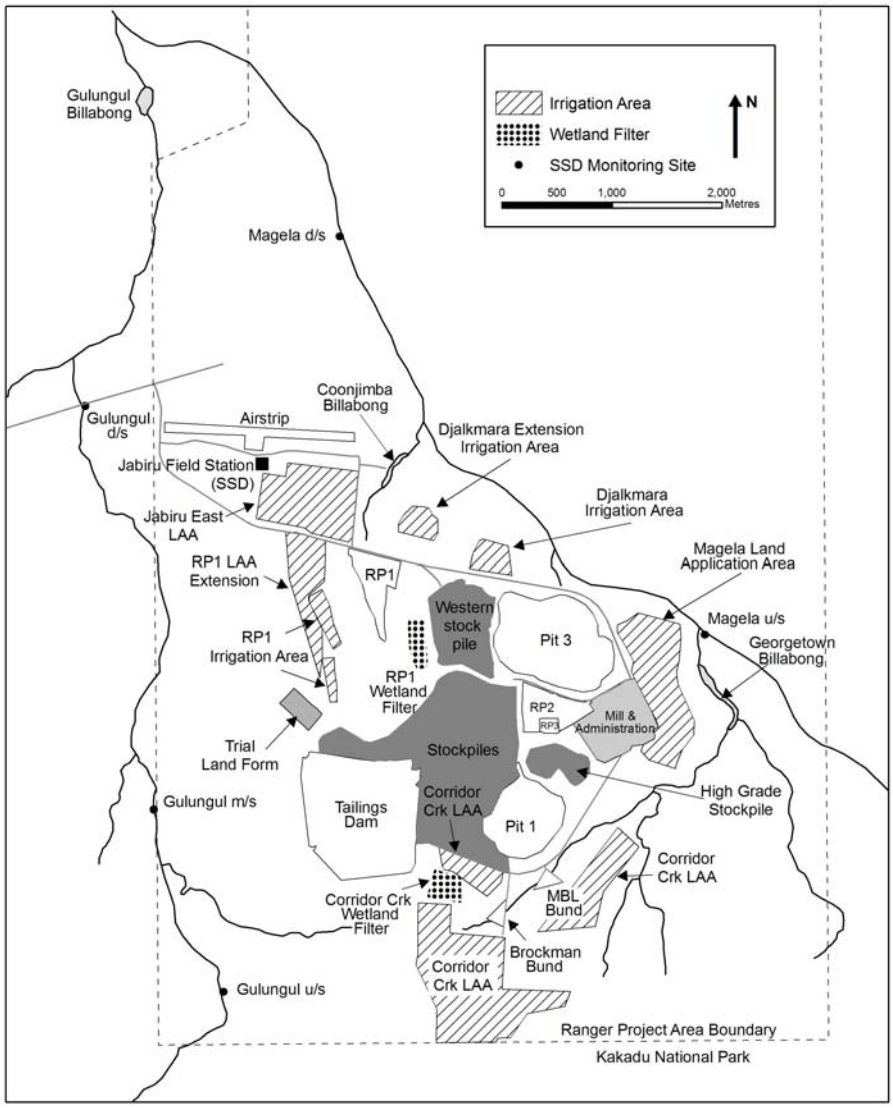


Figure 1 Schematic of Ranger mine site and surrounds showing location of the Magela u/s and Magela d/s monitoring sites used for wet season water quality and toxicity monitoring

1.3 Principle of the test

The test measures effects of wet season water quality on egg production by the freshwater snail, *Amerianna cumingi* (Mollusca, Gastropoda). The snail egg production test was originally developed to run in conjunction with an extended early life history test (embryo-juvenile survival test)[2]. However, the equivalent (at least) toxicological sensitivity of the egg production test, together with its reliability, ease of end-point response measurement and

[2] When conducted, the embryo-juvenile survival test is a continuation of the egg production test, with removal of adults and using some of the egg masses produced in that test to examine embryonic development and hatching success over a further 10–12 day exposure period. Further information on this test is contained in the Operational manual.

robustness under field testing conditions, has meant that this method is the only one that has been routinely used since 1991.

For the egg production test, pairs of snails of similar shell size are exposed for four days in test chambers held in floating containers in the creek upstream of the minesite (control water) and downstream of the minesite (exposed water). During this time, the snails lay 'egg masses' upon the walls of the test chambers. An 'egg mass' refers to a discrete batch of eggs that is surrounded by a gelatinous coat. Each egg lies in its own capsule within the gelatinous mass.

At the end of the four-day test period, the number of eggs produced at each site is counted. These values are compared between sites, and with values obtained in previous tests. Tests are conducted every second week (fortnightly) during the wet season, commencing shortly after first continuous flow is established in Magela Ck and continuing on to include approximately the first month of recessional flows. At this frequency, typically about 7 or 8 tests are completed during each wet season.

2 Experimental design

2.1 Statistical design

This test is based on principles of a Before-After-Control-Impact-Paired differences (BACIP) design described by Stewart-Oaten et al (1986, 1992). The test results are assessed by comparing data from a 'baseline' time series collected *before*, with data obtained *after* suspected contamination by mine wastewater or some other 'event' or a particular period of interest. Typically, the period of interest pertains to the tests conducted over the most recently-completed wet season. In future, the comparison might be between test results obtained after rehabilitation of the Ranger minesite with results gathered during the operational phase of mining. Egg production data are collected simultaneously at two sites, one located upstream (*control*) and the other downstream (*test* or *'exposed'* site) of potential inputs of contaminants from the Ranger minesite.

The water exposure system at each location is duplicated to detect effects that might arise from variation in the function of the equipment (including vulnerability to natural disturbances) and spatial variation in water quality. Thus at each site there are two floating containers holding the snail test chambers, each container located in a position that reduces as far as possible any significant spatial variations in water quality (eg mine contaminant gradients associated with mixing zones) and is independent of the other in terms of possible flow interference by one container upon another. Each duplicate floating container holds 8 replicate egg-laying chambers, and within each egg-laying chamber, there is a pair of snails. The overall experimental design is shown in Figure 2.

The BACIP design uses a form of temporal replication. The *difference* between sampled responses at the *paired* sites (upstream-minus-downstream) at any one time is regarded as a replicate observation. The means of sets of differences between the two sites, before and after an event or period of interest, are compared using the first factor (BA) of a two-factor ANOVA (Analysis of Variance) model, explained in greater detail below. If significant differences are found to occur between two periods within a time-series of paired-site difference values, this is taken to be a result of a change in water quality, which may have occurred as a result of inputs from the minesite. Continuous electrical conductivity (EC) and turbidity data (collected since the 2005–06 wet season) obtained at each location during the test, as well as possible event-based (SSD) or regular weekly (ERA) water chemistry grab data (for Mg and U

concentrations) provide the physicochemical evidence to assess if there has been a significant change in water quality.

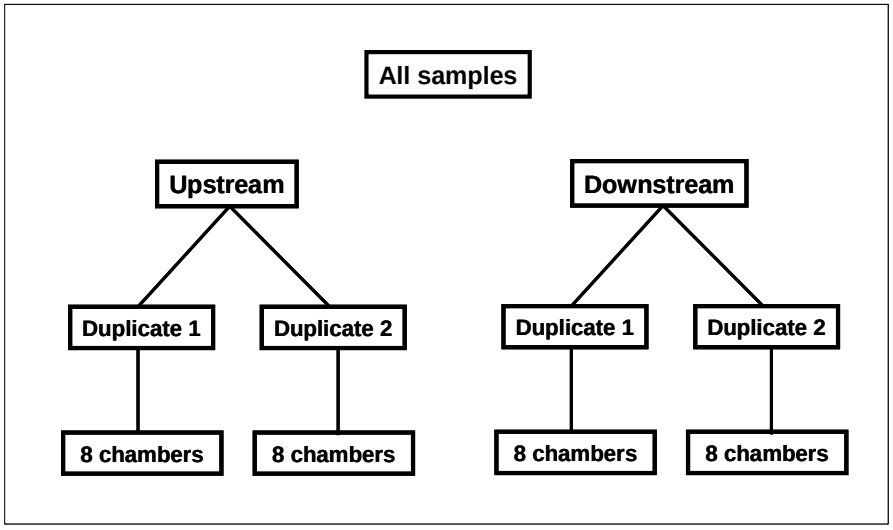


Figure 2 In situ toxicity monitoring design for the snail reproduction test

The mainstay of data analysis for change detection using this toxicity monitoring technique is based upon classical hypothesis testing. The original BACIP design described by Stewart-Oaten et al (1986, 1992) used a Student's *t*-test to compare the means of sets of differences between the two sites, before and after an event or period of interest. Current statistical testing of toxicity monitoring data has been extended to a two-factor ANOVA, with Before/After (BA; fixed) and Season (random, and nested within BA because different wet seasons/years are sampled before and after the event, or between the periods of interest) as factors. Thus, the particular ANOVA model used here allows variation amongst years (or wet seasons) and among tests (or 'runs') within a wet season to be estimated separately.

Some comment is required on the choice of Years(BA) as a *random* factor since there is debate in the literature about whether 'time' in ANOVA designs should be assigned as 'fixed' or 'random' (see Quinn & Keough 2002). Although the seasons are a sequential (and not random) sample, the primary aim of this analysis is to answer the question: Does the magnitude of any change from before to after exceed that expected, given the natural variation from year to year? Treating Season(BA) as a random factor addresses this question because seasons are not selected on the basis of particular characteristics they possess that may influence the response. While the assignment of time as a random factor would likely lead to a more conservative (less powerful) test (compared to fixed-factor designation), it is also worth noting that the BA test performed in this situation is comparable to the Student *t*-test method recommended by Stewart-Oaten et al (1986), authors of the original BACIP analysis method, and adopted routinely in numerous subsequent studies, where 'Seasons' (or time) are also treated as random.

In the ANOVA model described above, while the first factor (BA) serves the same function as the *t*-test, the second factor (Season) can be used to determine whether, within the Before and After periods, any set of difference values for a wet season are significantly different. Though this latter test may provide no more additional information in the case of a comparison of the (current) season of interest versus all previous wet seasons, in a comparison of several 'before' and 'after' seasons that are of particular interest, it can potentially identify variability among test responses. Further analysis of the season(BA) factor (using Tukey's pairwise comparison)

would then determine if significant differences amongst seasons occur only in the 'after' period (variability) that could indicate impact, even though mean difference values before and after are not significantly different. An additional advantage of the ANOVA is that it is generally a more efficient test with respect to simplicity in data preparation and testing of data assumptions.

In the absence of systematic seasonal changes in mean difference values, the ANOVA and *t*-test will have similar, and in some cases, equal statistical power. If there are systematic seasonal changes in mean differences (ie 'seasons' factor significant), then the *t*-test is invalid and the ANOVA approach is required to estimate, and account for, these changes.

The model for the ANOVA is: BA + Season (BA).

The ANOVA table is as follows (b = number of seasons before; a = number of seasons after; N = total number of replicates, in this case toxicity monitoring tests):

Source	**df**	**F**
BA	1	$MS_{BA}/MS_{Season\ (BA)}$
Season (BA)	$(b-1)+(a-1)$	$MS_{Season\ (BA)}/MS_{Error}$
Error	$N-(b+a)$	
Total	$N-1$	

Control charting is a complementary technique to the classical hypothesis testing approach described above and is used to detect at an early stage, shifts or trend away from a 'target' value for the paired-site difference values. Specifically, the CUSUM method is applied to toxicity monitoring difference data, with a chart displaying the cumulative sums (CUSUMs) of the deviations of each test difference value from the target difference value (in this case, of zero). CUSUM charts include control limits, which for industrial applications is conventionally set at ± 3 or 4 standard deviations (SD) from the target value, to determine when an 'out-of-control' situation has arisen. If drift or trend are not evident ('within control'), the test points on the chart should fluctuate randomly around zero. However, if a trend develops in either direction and ,in the extreme situation, observations move outside the control limits, this is taken as evidence that the 'process' mean is shifting and hence triggers an investigation of possible causes.

The liberal control limits (± 3 or 4 SD) typically applied for process control applications , provide for very small Type I errors, and are necessary for manufacturing industries to prevent excessive false triggering. For environmental protection purposes, it is important that real (mine-related) changes do not pass undetected and hence it is important that Type II error is minimised as far as possible. This may be achieved by increasing the Type I error rate which in this case requires a reduction in the control limits. Prof David Fox (Environmental statistician, Melbourne University, pers comm) has recommended a limit of ± 2 SD, consistent with similar effects sizes recommended in ANZECC & ARMCANZ (2000).

2.2 Hypotheses

Control charting and more traditional hypothesis testing are used to assess whether or not, for the measured difference in egg production, there has been either (i) a shift or trend away from a 'target' value for the differences and towards (or outside of) a control limit (control charting), or (ii) a significant change in the difference values between two time periods of interest (hypothesis testing).

2.2.1 Control charting using CUSUM

Points representing the original paired site difference values on the CUSUM chart should fluctuate randomly around the target value (zero). If a trend develops in either direction (upwards or downwards) and/or the trend leads to exceedance of the control limits (± 2 SD), this is taken as evidence that the process mean has shifted, eliciting investigation. Hypotheses based around both (i) trend, and (ii) control limit exceedance, may be expressed, respectively, as:

(i) H_0: the values of the cumulative sum (CUSUM) of the deviations of (paired-site) test difference values exhibit no systematic patterns or trends across consecutive tests over a period of interest; and

(ii) H_0: for a given toxicity test result, the cumulative sum (CUSUM) of the deviations of this and preceding (paired-site) test difference values from the target difference value does not exceed the control limits (± 2 SD).

2.2.2 Classical hypothesis testing

In general terms, the test is designed to evaluate the primary null hypothesis (ANOVA BA factor) that there has been no change in snail egg production at the downstream exposed site, relative to the upstream control site, between two time periods of interest (eg before and after a possible impact event), between the current wet season results and those from previous wet seasons, and before and after mine rehabilitation etc. Specifically, the null hypothesis may be expressed as:

H_0: mean difference in paired-site snail egg production before the event (or period of interest) equals mean difference after the event (or period of interest).

If the test is significant, then the null hypothesis is rejected: the mean difference after the event differs (is either smaller or larger) from that before the event.

The second factor (Season) can be used to determine whether, within the Before and After periods, any set of difference values for a wet season are significantly different, even if a test of the primary hypothesis shows that the mean difference values before and after the event (or period of interest) are not significantly different. The null hypothesis may be expressed as:

H_0: differences amongst the paired-site difference values for particular wet seasons within the Before and After periods are the same.

If the test is significant, further analysis of the season (BA) factor, using Tukey's pairwise comparison, is used to determine if significant differences occur only in the 'After' period (variability) that could indicate impact.

The biological significance of any observed change or trend (in relation to mining activities) is assessed and/or investigated further.

3 Test procedures

3.1 Test organism

The test species, *Amerianna cumingi* (Mollusca, Gastropoda), occurs in lentic floodplain habitats of the Alligator Rivers Region. Important aspects of the biology of the snail are summarised in an appendix of the Operational manual. All specimens required for testing are taken from Jabiru field station (JFS) laboratory stocks which have been cultured from a sample of animals collected from the Magela Creek floodplain. Snails were collected originally in 1986 and laboratory stocks were supplemented with new field stock in April 2000 and September 2010. (Comparative performance in toxicity monitoring tests of pre-

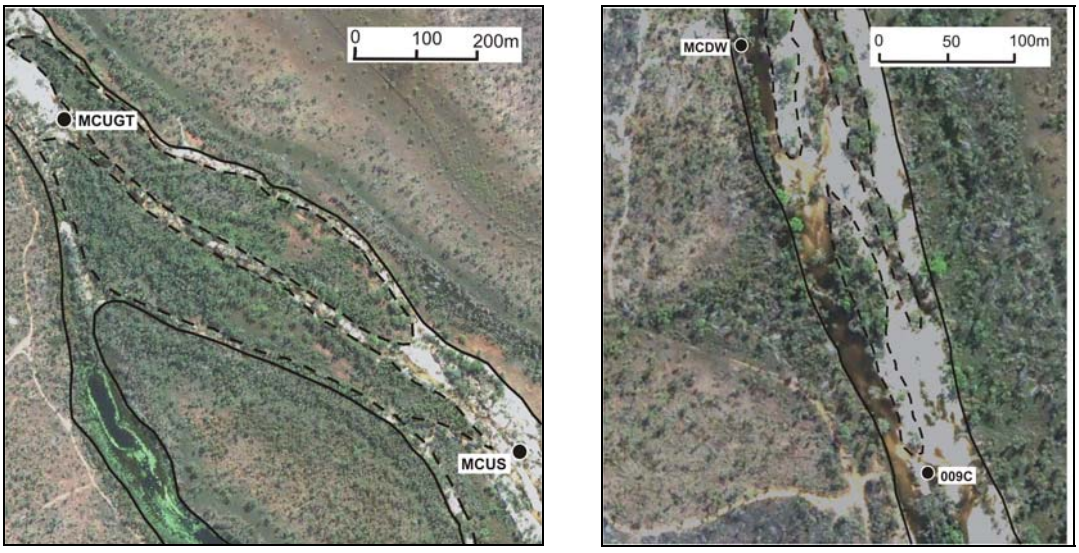
2000 and new 2000 snails stocks is reported in Supervising Scientist (2001, pp 49–50).) Adult snails of between 10–12.9 mm shell length are used for testing, this being the optimal size for egg laying. Larger animals (>13 mm) may be senescent (Jones 1992).

3.2 Field sites, infrastructure and equipment

3.2.1 Field monitoring sites

In situ toxicity monitoring is conducted at designated monitoring sites in the creek, upstream and downstream of potential input of contaminants from the Ranger minesite. The same sites are used for SSD's grab and continuous water chemistry monitoring programs. To ensure the validity of tests, careful management of field equipment and mooring pontoons is required. Back-up equipment is available in the event of loss through vandalism or natural flood disturbances. Full details of the field sampling infrastructure and operational and maintenance procedures are outlined in the Operational manual.

The physical configurations of the stream channels at the upstream and downstream monitoring locations in Magela Creek are shown in Figure 3. The upstream control site is located in the main channel of Magela Creek, upstream of any mine influence. It is 90 m upstream of the Ranger mine release pipe (the latter located adjacent to ERA's gauging station, MG001) and 50 m laterally (but separated by a peninsula of land) from the confluence of the outlet of Georgetown Billabong with Magela Creek..



A Upstream monitoring site on Magela Creek

B Downstream monitoring site on Magela Creek

Figure 3 Upstream and downstream monitoring sites used in the SSD's water chemistry (grab sampling and continuous) and toxicity monitoring programs. Channel boundaries are indicated by the continuous or broken (water-level-dependent) lines.

The downstream 'exposed' site is located in the west channel of Magela Creek, 370 m downstream of Gauging Station GS8210009, and 5.1 km downstream of the control site. The water quality characteristics of the two locations are described in Brazier & Humphrey (2009).

The location coordinates for the control and exposed sites are provided as follows (GPS accuracy to 7 m):

Site	Latitude (S)	Longitude (E)
Upstream control (MCUGT, Figure 2A)	12° 40.472'	132° 55.859'
Downstream exposed (west bank) (MCDW, Figure 2B)	12° 38.312'	132° 53.962'

Independent floating containers holding the snail test chambers (Figure 4) are tethered to floating pontoons at each site (see Figures 1 and 3 for location). The containers are adjacent to one another to reduce spatial variations in water quality, but sufficiently apart that there is no flow interference between the containers (section 2.1).

Figure 4 Picture of Magela Creek upstream (control) toxicity monitoring location showing test containers trailing from floating pontoon bearing water quality instrumentation

3.2.2 Snail test containers

Each duplicate floating container holds 8 replicate egg-laying chambers. Containers are plastic rectangular bins (~50 L) with tie-down lids (see Figure 4). Circular, mesh-covered holes on the side of each container provide flow-through of creek waters while the rim of each container sits inside a floating frame. The floating frame is tethered to a floating pontoon (Figure 4).

3.2.3 Egg-laying chambers

Sixteen egg-laying chambers are required per site (treatment), 32 in total for each in situ test. Each of the chambers consists of an open-ended cylinder of clear polycarbonate (70 mm long, 50 mm external diameter) (Figure 5). Coarse mesh-screen ‘stoppers’ are inserted into each end of the chamber (Figure 5) to retain the snails while allowing a flow-through of water.

3.2.4 Ancillary equipment

A description of ancillary equipment used in the toxicity monitoring tests, including Vernier calipers, binocular dissecting microscope, transport containers, lettuce used to feed snails, lettuce cutter and ‘anti-scratch’ squares to protect the egg-laying chambers, is provided in the Operational manual.

3.3 Test animals

3.3.1 Husbandry of laboratory snail stock

Ensuring adequate health and numbers of snails for toxicity monitoring, requires close attention to the husbandry of *A. cumingi*. The equipment, facilities and procedures required for successful rearing are outlined in the Operational manual.

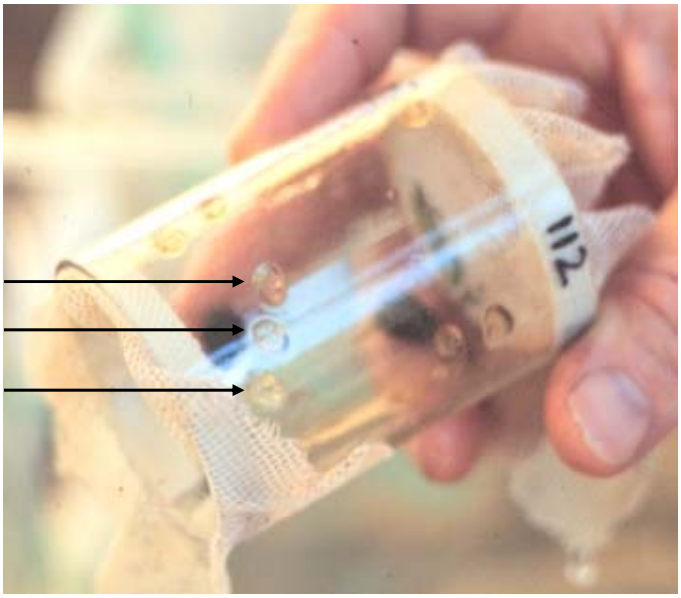


Figure 5 Egg-laying chamber showing mesh screens, circlips and identification code, and attached egg masses (denoted by arrows). Snail pair is shown as dark out-of-focus images on back wall of the chamber.

Adequacy of snail stocks should be assessed at least 2 months prior to commencement of testing each wet season. This allows sufficient time for set up of new cultures if numbers of young adults are likely to be insufficient to commence wet season testing.

Supplementation of JFS laboratory stocks with new wild snails from Magela floodplain should be programmed for 5 year intervals to minimise the risk of inbreeding that could potentially affect snail sensitivity (see Supervising Scientist 2001, pp 49–50).

3.3.2 Suitability of snails for testing

Snails are obtained from JFS laboratory stocks of *A. cumingi*. Careful handling is required to avoid any damage to their shells prior to deployment of the snails in the field.

All test animals must be free of overt signs of disease or shell damage. If there is any doubt about the health of the snails they can be weighed prior to testing and their weights compared with those of similar-sized healthy individuals. For the size range of snails tested (10–12.9 mm shell length) corresponding weights should be between the range 250–500 mg. Snail pairs which fail to lay egg-cases after a four-day exposure to natural (control) creek waters are regarded as abnormal (see also section 5.1). Shell or internal damage to one or both of the snails, arising from mis-handling, is often found to be the cause and this can quickly lead to the death of animals. Snails must, therefore, be handled carefully during all stages of testing. Further details on the handling of animals are provided in the Operational manual. More extensive failure of snails to produce healthy egg cases may indicate presence of disease (eg trematode parasitism). Should the latter be observed, new stocks from the field may be required (see Operational manual).

Snails used in a toxicity monitoring test must not be used in subsequent tests. Snails exposed to control creek waters in previous tests may be used only as stock for further snail production at the JFS laboratory. Snails exposed to creek waters downstream of Ranger are euthanaised at the completion of the test.

3.4 Pre-wet season testing regime

3.4.1 Infrastructure maintenance

Maintenance and upgrade of the aquaculture facilities must be carried out in the dry season and be completed by late September each year, ie well before the likely start of intensive snail culturing. Routine maintenance is outlined in the Operational manual.

3.4.2 Equipment preparation

Equipment inventory

The condition and availability of equipment must be checked at least one month prior to any possible testing. This allows time for purchasing, or for manufacturers or contractors to replace missing or damaged equipment.

Cleaning test equipment

Prior to initial use in the wet season, all test containers, egg chambers, nylon mesh, anti-scratch squares and circlips must be cleaned and rinsed.

3.5 Wet season testing regime

3.5.1 Timing and duration of toxicity monitoring during the wet season

Toxicity monitoring is conducted every-other-week (fortnightly) for the period of 'significant' wet season flow in Magela Creek. Most typically, first-flow commences at Gauging Station 8210009 (390 m upstream of the toxicity monitoring exposed site) sometime in December. In this scenario, the first test for the wet season should be conducted in the week after first-flows though the actual commencement time for testing is ultimately determined by water levels. False starts to flow in the creek which are more typical of early rains and subsequent creek flows in October and/or November, may mean a delay in testing until water levels are sufficiently high to sustain flow. Toxicity monitoring would not normally be conducted once flow over the spillway of Ranger Retention Pond 1 (the major source of solutes into Magela Ck) has ceased for the wet season. This discharge typically ceases in late April.

3.5.2 Test environment

The culturing of snails to be used in the tests (Operational manual), and all manipulation and testing should be carried out on premises or areas free from harmful vapours and dusts, and undue disturbance. No cigarette smoking or consumption of food is allowed within the aquaculture facility, or near the pontoons, and smokers should wash their hands before handling experimental equipment and animals.

The environment in the test containers must be as uniform as possible. The main conditions that could vary are light exposure, temperature and water exchange rate.

3.5.3 Test set up

Snail selection

Snails for each test are collected from JFS laboratory stocks early on the morning of day 1 of the test. Sixty-four snails are required for each test. A small number of extra snails (2 or 3) are required for replacement of dead or ailing individuals that may be observed in the field prior to deployment in the in situ floating containers.

Snails in the range 10–12.9 mm shell length (see Figure 6) are collected and sorted into three 1 mm size classes: 10–10.9, 11–11.9 and 12–12.9 mm. Gentle handling of snails is necessary to prevent damage to snail shells during collection and measurement. Snail selection is

assisted by collecting from cohorts of known age. This may also help to avoid selection of animals that are old (and possibly senescent) for their size. Shell length is taken as the distance between the apical tip and the shoulder (Figure 6).

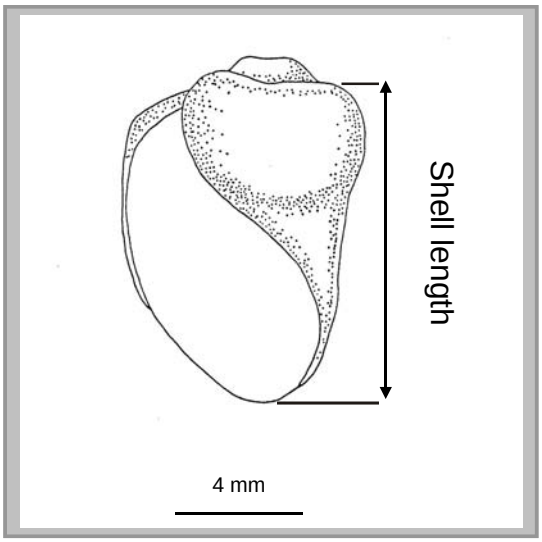


Figure 6 Ventral view of the shell of *Amerianna cumingi* (after Jones 1992)

Each treatment duplicate is allocated 8 pairs of snails from the stock. This makes thirty-two snails required per treatment and a total of 64 snails required for each test.

Set-up of egg-laying chambers and allocation of snails

Size classes must be distributed evenly amongst, and between the duplicates of, the different treatments. To ensure this, the size allocation procedures outlined in the Operational manual must be carried out for each group of 8 egg-laying chambers that will be assigned to each treatment duplicate. Using these procedures, the following size pairs are present: 10 and 11 mm [x 3 pairs], 10 and 12 mm [x 2], 11 and 12 mm [x 3].

All effort must be made in a test to use this allocation of size classes of snails for treatment duplicates. Should a situation arise, however, where there are insufficient numbers of snails of a particular size class, additional snails from one of the other two size classes may be used, providing the overall mean size of snails tested lies in the range 11–11.9 mm. As before, size classes must be distributed evenly amongst, and between the duplicates of, the different treatments. This may be achieved using an appropriate modification of the procedure of allocation described previously. No snails outside of the size range 10–12.9 mm may be used in toxicity monitoring tests.

Eight 20 mm diameter discs of freshly-cut, outer lettuce leaf, washed in demineralised water are added to each egg-laying chamber containing the snail pair. The open end of the chamber is then covered by nylon mesh to retain the snails. The groups of 8 egg-laying chambers with their enclosed snails are carefully transported to the field in containers holding laboratory snail stock-tank water.

Field set-up of test containers and acclimation

On the morning of commencement of each test, the duplicate containers are cleaned to remove any deposited sediment or detritus, or algal build-up from the container walls or nylon mesh covering the flow-through portal holes.

At each site, the 8 egg-laying chambers and enclosed snail pairs of each treatment duplicate are gradually acclimated to creek water. Creek water is added to the snail transport containers to make a 50:50 mixture of creek and snail pond water. Each container is then left for 30 minutes after which time the containers are topped up with creek water to stand for another 30 minutes. The egg-laying chambers are then transferred to test containers containing 100% creek water. All air bubbles must be removed from each chamber as these reduce the available egg-laying surface area.

The test is deemed to have commenced when all egg-laying chambers have been placed in the test containers, the lids are locked in place and the test containers are positioned appropriately. Commencement of the test at each site must be within one hour of each other to ensure equal exposure time and exposure to similar stream conditions, thereby minimising the chance that more or less eggs are laid at one site by virtue of a longer or shorter period in the creek.

Assuming no interference by debris to the water entry portals located on the sides of the duplicate floating containers, then flow rate through the containers is dictated by actual discharge rates of the creek itself[3]. It is important that in any particular test (within and between different water treatments) each test container receives a similar flow rate, acknowledging that amongst different tests and even within the same test, flow rates through the containers will vary, depending upon creek flow conditions. In practice, comparable flow rates amongst containers will best be achieved by placing these in positions in the creek of as similar flow as possible.

3.5.4 Observations made during the test

The test runs for four days (96-hours). During this test period, the only additional requirements are the acquisition of accompanying hydrological and physico-chemical data. It is important that for proper interpretation of toxicity monitoring data that water quality data are available for each testing period. To this end, the integration of the toxicity monitoring program with other water quality monitoring programs on Magela Creek is essential. This information comes from one of several data sources:

1. SSD's continuous monitoring program conducted at the same sites (as toxicity monitoring) on Magela Creek. Continuous monitoring of water temperature, electrical conductivity (EC, an excellent surrogate for $MgSO_4$), pH and turbidity is conducted at each site. (Dissolved oxygen concentrations are measured continuously in Magela Creek waters but this variable is not regarded as important because levels in creek waters are invariably at or above their air-saturation value.)
2. SSD discontinued its routine weekly grab sampling program in the 2010–11 wet season. The only laboratory measurements for water chemistry variables are derived from event-based samples collected at the same sites (as continuous and toxicity monitoring). These samples are triggered at 'high' EC and turbidity thresholds and hence the frequency of sampling cannot be predicted nor guaranteed to coincide with the period over which a toxicity monitoring test is being conducted. Event-based samples are analysed for U[4], Mg, Mn, SO_4 and Ca.

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[3] At the time of completing this report, only one measurement had been made, at the upstream station, where a flow rate of 1.82 L/minute was measured at a creek flow rate of approximately 200 cumecs.

[4] As discussed in section 6.2 item C1, the concentrations of U measured in Magela Creek downstream of Ranger are generally never more than two orders of magnitude below those concentrations known to elicit ecotoxicological responses in *A cumingi*. To this end, it is highly unlikely under normal operational waste water discharges from the mine site that U would ever contribute as a potentially important explanatory variable for the snail egg production response.

3. ERA collects water samples for chemical analysis on a weekly basis, from near the same sites as SSD's continuous and toxicity monitoring sites. These samples are analysed for U, Mg, Mn, SO_4 and Ca.
4. Since the 2010–11 wet season, an integrated measure of suspended organic carbon available to snails (as a potential food source) was being derived from detritus settling in, and collected from, open plastic containers held in each duplicate test container for the duration of each toxicity monitoring test. At the time of publication of this protocol, the relationship (if any) between these data and snail egg numbers had not been examined closely.

Toxicity monitoring tests typically commence on a Monday and finish on a Friday. While there is flexibility in the start and finish days, tests that coincide with the week day that ERA collects samples for its routine weekly water chemistry program (see 3 above) are potentially advantaged by accompanying water chemistry data (albeit, a spot sample in time).

3.5.5 Final day – snail removal and egg counts

After four days (96-h) exposure, counts are made of eggs and egg masses deposited on the inner surface of the chambers. This examination may occur in the field though more typically it is conducted at the JFS laboratory. For JFS laboratory examination, each chamber must be transported carefully from the field in a container holding a sufficient volume of its respective treatment water to cover the chamber. Once at the laboratory, the circlips, mesh and snails are removed from the egg laying-chambers. Considerable care must be taken in dislodging snails from the inner walls of the chambers to prevent damage of the egg masses. For either field or JFS laboratory examination, it is important that snails from all treatments are removed from chambers within one hour of each other (see rationale above under 'Field set-up of test containers and acclimation').

Instructions on how the counts are conducted (number of operators and cross-check procedures) are described in section 3.7 below ('Final-day egg counts').

For (dissecting) microscopic examination, either in the field or JFS laboratory, each chamber is placed on its side and rotated systematically from a reference point (eg the chamber identification code) to conduct the counts. Two separate counts (full rotations of the chamber) are made. If there is a discrepancy between the counts, additional counts are made until consecutive same-counts are reached. The numbers of egg masses and number of viable embryos per egg mass attached to the inner wall of each chamber for the four-day period are recorded on datasheets (Operational manual).

At this stage, egg masses that contain no viable embryos are included in the counts. Otherwise, unusual and anomalous egg masses and embryos (eg absence of cell capsules, twin embryos in a single cell, damaged egg masses) are noted. Photomicrographs in the Operational manual display these while accompanying narrative indicates how to note these observations in the data sheets.

3.5.6 Test clean-up

After each test, egg chambers, nylon mesh, circlips and anti-scratch squares are cleaned at the JFS laboratory using the steps outlined in the Operational manual:

3.6 Training and QA/QC procedures for snail egg production test

3.6.1 Use of trained staff

To ensure the validity of snail egg and egg mass counts, only trained staff must feed and handle the test organisms. At the start of each wet season, all trained staff need to review the protocol and field methods, ensuring consistency in all aspects of the snail test. It is a lengthy time period between the completion of wet season toxicity monitoring and commencement of the following season's testing and so (re-)familiarisation and training of new staff needs to commence in the dry season, well in advance of intended participation. Once wet season toxicity monitoring commences, training of new staff must formally encompass at least three consecutive early tests.

Damage to snail shells, inconsistent feeding and husbandry, introduction of toxicants (eg from cigarette smoking) and selection of unhealthy snails will reduce the quality of test results. Incorrect handling of egg-laying chambers in the field may also result in damaged egg masses, making egg counts difficult or impossible.

3.6.2 Training

Training needs to be conducted over a number of toxicity monitoring tests to ensure all possible sources of error have been identified and minimised to acceptable rates. Training should be conducted in stages to ensure its effectiveness. The level of training will vary, depending upon the complexity and/or importance of the task, and for this reason, tasks have been divided into components viz the sub-headings below. At all times, field procedures and check lists outlined in this protocol, and provided in more detail in the Operational manual, are to be followed.

Snail selection, handling and measurement

So that additional (pre-handling) of test organisms is minimised as far as possible, training can be conducted during the procedural steps of actual tests. Training of new staff is conducted in three stages, over three consecutive early wet season tests, thus:

1. Stage 1. Trainee must observe (only) the procedures conducted by a trained staff member (first of the three toxicity monitoring tests only). It is important that the snail measurement procedure is explained and key points in identifying unhealthy and damaged snails are demonstrated until the trainee is confident with, and understands, the procedure.
2. Stage 2. Trainee staff member selects and measures snails whilst under the close supervision of the instructor (each of the three toxicity monitoring tests). The trained staff member (instructor) ensures that measurements, handling to prevent damage to snails, and selection of healthy snails are consistent and correct.
3. Stage 3. Snail selection by the trainee is checked by trained staff (each of the three toxicity monitoring tests). A random selection of three snails from each of the three size classes is examined by the instructor to ensure they are within the correct size range and to ensure healthy specimens.

Should the trainer not be satisfied that any one of the protocol procedures is being followed correctly after the three consecutive toxicity monitoring tests, the training procedure continues until such time as all test procedures are being conducted correctly.

Egg counting

When training new staff, a minimum of three comparative egg counts (usually from three consecutive toxicity monitoring tests) must be conducted. Specifically, egg masses and numbers of eggs per egg mass from sixteen egg-laying chambers are counted on each of the three test occasions. Results are compared to those of a trained observer (making counts on the same egg-laying chambers), and any differences encountered are explained to the trainee to ensure a proper understanding of the protocol. Unusual and anomalous egg masses and embryos (eg absence of cell capsules, twin embryos in a single cell, damaged egg masses) are shown to the trainee, together with advice on what to record in the circumstances.

For the comparative counts, the QA/QC acceptance criteria described in the Operational manual for the egg counts are applied. The results of these checks are used by the trained observer (preferably Jabiru-based, but with at least two wet seasons' experience) to decide upon the intensity of comparative counts to be conducted after three tests.

3.6.3 Snail selection and size measurement

Snail selection and measurement are key components of the egg production test. Test organisms outside the designated size range may have reduced egg production or be senescent. Due to the ease in incorrectly measuring snail shell length and ease of damage to snails, staff training is required. Feeding lettuce to snails on the afternoon before a test set-up ensures snails are habituated to the diet they will be placed on during the test and facilitates snail collection as they are easily removed from the lettuce (ie the foot of the snail does not adhere strongly to this substrate). This helps to prevent damage to the snails.

Prior to the toxicity monitoring test period for a particular wet season, all trained staff must demonstrate consistency in handling, measurement and snail selection by way of comparative assessments conducted amongst staff.

3.6.4 Snail feeding – lettuce selection

Feeding of snails with lettuce discs must be consistent across all chambers to prevent any bias in the testing. Lettuce discs must come from the outer edge of the outer (greenest) leaves only. Veins in the leaf are avoided as these portions of the leaf are not palatable.

3.6.5 Final-day egg counts

As a matter of routine, two egg count observers are required for each test, each counting the eggs and egg masses from chambers exposed at one of the two monitoring sites. At the completion of their own counts (from 16 replicate chambers), each observer must conduct the following procedure on the egg-laying chambers from the other site (counts performed by the counterpart observer):

1 Counts the number of egg masses on all 16 chambers.

2 Ensures the number of egg masses tallies with the number of egg masses recorded (ie the primary observer has not missed an egg mass). If an egg mass has been missed, the egg count for the chamber in question has to be repeated.

3 Randomly selects three chambers and conducts egg mass and associated egg counts. If a discrepancy is encountered in the egg mass checks (from 2 above), this chamber should be included in the selection. For each of the chambers, there should be no greater than a 5% difference in the total egg count recorded by the two observers.

4 If acceptance criterion from 3 is not met, correct errors, then repeat this step using another three randomly-selected chambers, until error rates are acceptable.

Differences in counts encountered due to (infrequent) damaged or deformed egg masses may be overlooked due to the difficulty in estimating the number of eggs.

4 Data entry, storage and associated QA/QC

4.1 Data storage

All data relevant to snail egg production tests can be found in the SSD Explorer directory system. Further information about the databases, their fields and importation of data is contained in the Operational manual. Directory paths and relevant files are as follows:

4.1.1 Snail egg production data

Snail egg production data can be located in the Access database labelled 'Toxicity monitoring database 12012010 version'. This relational database is stored in SSDX Sharepoint under the directory pathway:

Supervising Scientist Division > SSDX > Environmental Impact of Mining - Monitoring and Assessment > Toxicity Monitoring > Databases > Current data

4.1.2 Water chemistry data

Since the 2008–09 wet season (commencement of in situ toxicity monitoring), all water chemistry general parameters and metals and solutes data have been obtained from SSD's water chemistry monitoring program for the corresponding periods (see water chemistry protocol for data storage details – Supervising Scientist Division 2011a).

Prior to 2008–09, water chemisty data were collected from the header tanks and test containers used for the historical creekside monitoring technique. Water chemistry data for the period 1992 to 2007 can be found in the SSDX sharepoint directory pathway:

Supervising Scientist Division > SSDX > Environmental Impact of Mining - Monitoring and Assessment > Toxicity Monitoring > Magela Creek CSM monitoring > CSM DATA > water quality

4.1.3 Continuous monitoring data for general physico-chemical parameters

At present, continuous monitoring data gathered since 2005 and pertaining to dissolved oxygen, water temperature, pH, electrical conductivity and turbidity are exported from the Hydstra database, which is managed by SSD's water chemisty monitoring team (see water chemistry protocol for data storage details – Supervising Scientist Division 2011a).

4.2 Data entry and QA/QC

Data are entered into the 'snail database' using the form 'SNEV1' (section 4.1.1). This form can also be used for data validation, where a sum query calculating the total eggs per chamber can be checked against the values recorded on the validated datasheets. All data entered into databases and spreadsheets, as well as critical calculations, are validated by a second person. Once the database is validated, the corresponding datasheets are marked 'Data validated', dated and signed.

5 Data analysis

As described in section 2.1, the BACIP design employed for toxicity monitoring in Magela Creek uses a two-factor ANOVA, with Before/After (BA) and Season (nested within BA) as

factors. The tests are used for the detection of intermittent or sustained and continuous effects arising from dispersion of mine wastewaters to the creek. Of primary interest, the means of sets of differences (in snail egg production) between the two sites, before and after an event or period of interest, are compared using the first factor (BA) of the ANOVA model. Egg production data from a time series of toxicity monitoring tests – specifically, the mean number of eggs laid per snail pair for the 16 replicate pairs of snails exposed to each treatment – are required for the analyses, with details and worked examples provided in the Operational manual. Analyses of the test data for are conducted as follows:

5.1 Rejection of zero values

For results of each test, use a query in the Access database to tabulate the total number of eggs laid by each of the 16 replicate pairs of snails over the four-day exposure period. Reject any values for which a zero is recorded.

Failure of snails to lay eggs after a four-day exposure to natural waters, is an uncommon and abnormal response indicating unhealthy test organisms (see section 3.3). Otherwise, absence of *any* egg production has only been observed after snails have been exposed to very high concentrations of the dominant Ranger mine water constituents, ie for U, ~500 µg/L (Lewis 1992, Hogan et al 2010) and for Mg, ~600 mg/L (van Dam et al 2010). Under normal mine wastewater operations, these contaminant levels are never likely to occur in the field, even in mixing zone waters, and so inclusion of these (zero egg number) data would not be relevant to current field monitoring applications.

5.2 Testing of ANOVA (and control charting) assumptions

ANOVA analyses are conducted on the difference values arising from the tests, a single value for each test being derived from the difference between the mean number of eggs laid per snail pair for the 16 replicate pairs of snails exposed to each treatment, upstream and downstream. It is important to check that the full dataset to be analysed (difference values) conform to underlying assumptions of ANOVA, including normality, homogeneity of variance, additivity and independence (eg Stewart-Oaten et al 1986, Sokal & Rohlf 1995). For control charting, data must also conform to underlying assumptions of normality and independence (Gibbons et al 2009).

Assumptions of **normality** and **equal variances** are checked graphically, as recommended by McGuinness (2002). For this, plots of the *residuals* or *errors* (ie the difference between an observation and the mean for the group) are examined. A worked example of this procedure is provided in the Operational manual using MINITAB software. Both assumptions are invariably met in creekside data so far examined – since 1992. (ANOVA is considered to be robust to these assumptions, particularly with large datasets. Even if the assumptions are not met for a particular test, absolute compliance with these assumptions is not an essential requirement.)

Additivity refers to constancy of the differences (Stewart-Oaten et al 1986), ie the difference values are not proportional in magnitude to the average values of egg production calculated between the two sites. (If non-additivity was observed, data transformations may be necessary.) Humphrey et al (1995) and subsequent analyses have shown that this assumption has been met in toxicity monitoring data arising in Magela Creek since 1992.

If errors are arranged in the temporal order of the data collection, they are expected to succeed each other in a random sequence, ie the errors are **independent**. Departure from independence would be indicated in either a long sequence of large positive values followed

by an equally long sequence of negative values, ie *positive autocorrelation*, or positive and negative values alternating with regularity, ie *negative autocorrelation*. The plot of residuals versus observation order (in MINITAB) may be used as an initial screening assessment for lack of independence, with formal testing conducted using the von Neumann test as detailed in Sokal & Rohlf (1995, pp 394–396). The latter test is demonstrated in the Operational manual where it is shown that difference data (from 1992 to 2010) are not serially correlated.

Checks of the ANOVA and control charting data assumptions should be made annually.

5.3 Time series plot and statistical analysis of egg production data

The mean number of eggs laid per snail pair for each treatment location in the spreadsheet file containing corresponding data for tests previously conducted (since 1992, file details provided in Operational manual). The difference between the two sites (control site minus exposed site) is calculated.

The time series of these data, mean egg production at control and exposed sites and the difference between these as a time series, are plotted (Figure 7) (details provided in Operational manual). CUSUM and two-factor ANOVA analyses are conducted on the difference data (in this case using MINITAB software). By way of example, application of both analysis types to the toxicity monitoring results arising from the 2009–10 wet season is demonstrated below.

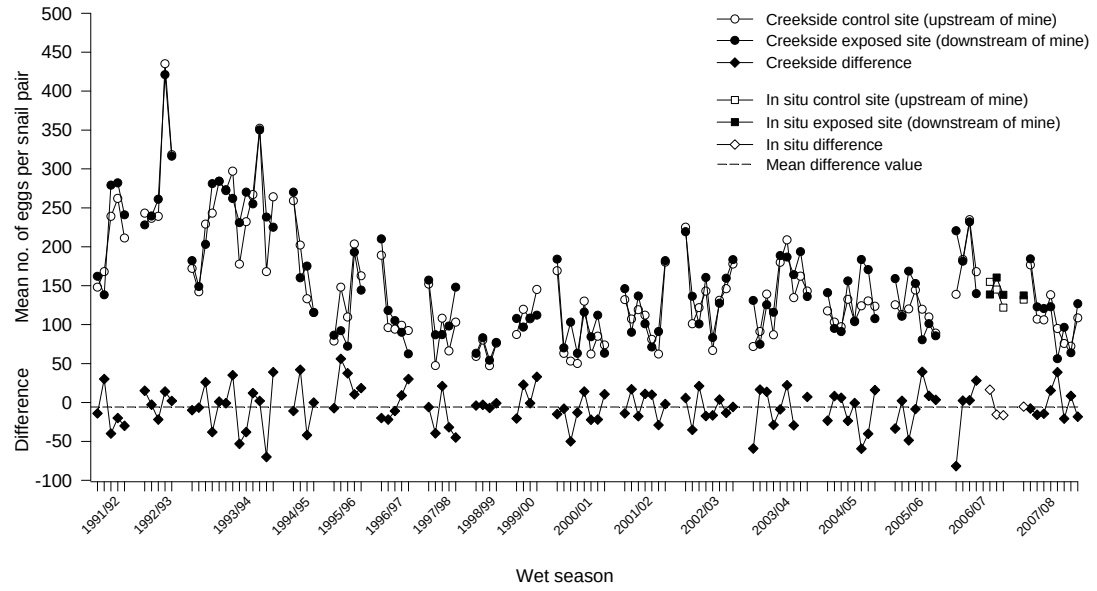
5.3.1 Control charting using CUSUM

A plot of the CUSUM chart for the complete time series of (upstream-downstream) difference values from 1992 to 2010 is shown in Figure 8.

CUSUM values above zero indicate higher upstream egg production relative to downstream while CUSUM values less than zero represent higher downstream egg production relative to upstream. A period of extended higher upstream egg production was observed in the 1995–96 wet season period (between test samples 27 and 40). More recently, two periods of extended higher downstream egg production occurred, between the 2003–04 and 2005–06 wet seasons (between test samples 75 and 93) and in the 2009–10 wet season (from test sample 118) (Figure 8).

The CUSUM values representing the higher upstream egg production observed during the 1995–96 wet season exceeded the calculated Upper Control chart Limit (UCL) for three consecutive tests (Figure 8). The two extended periods when egg production was consistently higher at the downstream site (2003–04 to 2005–06, and 2009–10 wet seasons) led in both occasions to ‘out-of-control’ situations where the Lower Control chart Limit (LCL) was exceeded. The increasing tendency for egg production to be higher downstream of the mine, in particular, and for observations to move outside the control limits on these occasions, represent rejection of the null hypotheses posed for CUSUM control charting (section 2.2.1), and suggests that the ‘process’ mean may be shifting. This condition indicates a more detailed assessment or investigation of possible ecological significance and causes may be needed (see section 6).

A



B

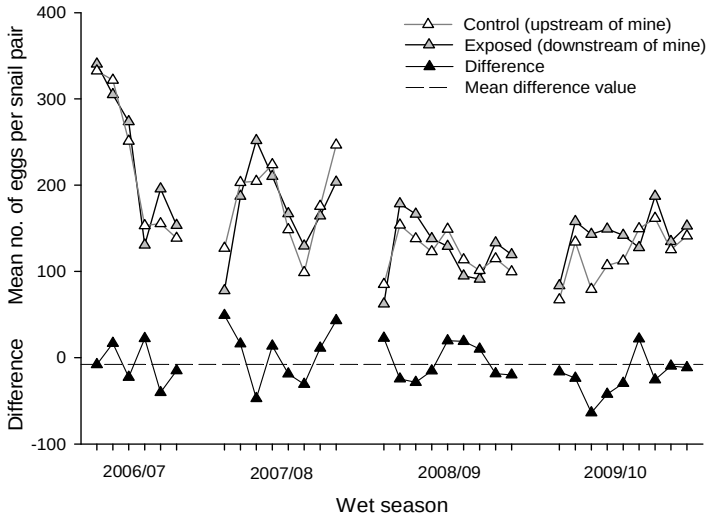


Figure 7 Time-series of snail egg production data from toxicity monitoring tests conducted in Magela Creek using A: (mostly) creekside tests, and B: in situ tests. Difference = upstream minus downstream egg counts.

5.3.2 ANOVA testing

The difference values are compared statistically for different parts of the time-series using the two-factor ANOVA from section 2.1. In particular and typically, difference data for the wet season of interest are compared with those from previous years; if they differ significantly, it may indicate a mine-related change.

Applying the two-factor ANOVA from section 2.1 above to the toxicity monitoring results arising from the 2009–10 wet season, upstream-downstream difference values for snail egg production data were found to differ significantly from difference values measured in previous wet seasons ($p < 0.05$), while no differences were observed among the difference values for

particular wet seasons within the Before (pre-2008) and After periods (p = 0.733). The MINITAB output for these results is shown below (Table 1), with details of how to set up the ANOVA model and run the analysis in MINITAB provided in the Operational manual.

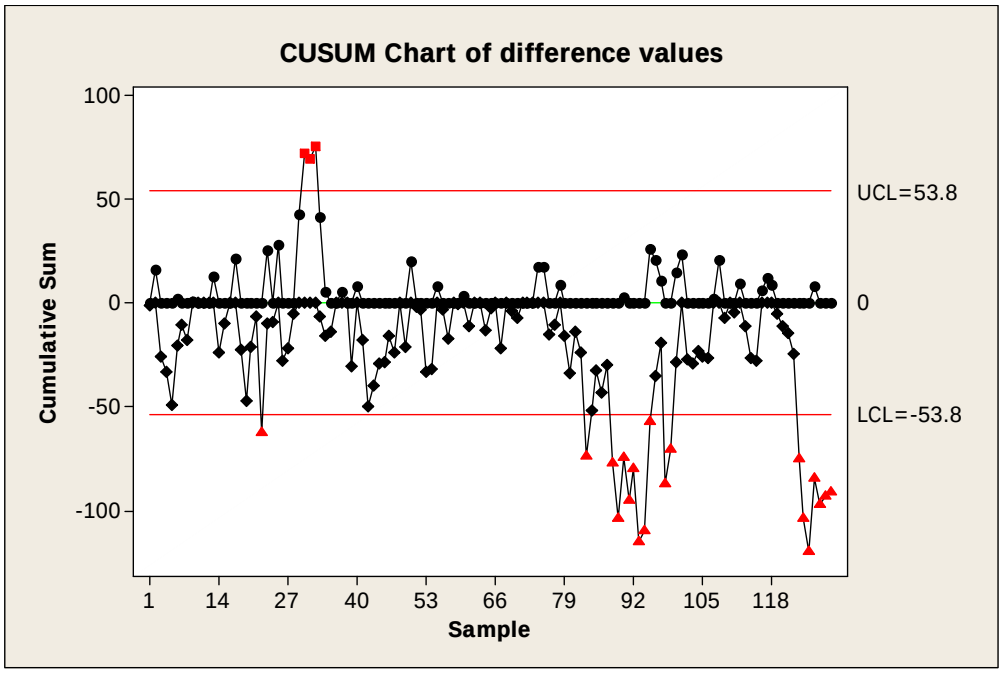


Figure 8 CUSUM plot of the time series of toxicity monitoring difference values, 1992 to 2010. Control limits (CL) set at ± 2 standard deviations.

Table 1 Results of Two-factor ANOVA comparing egg production difference responses between 2009–10 wet season and previous wet seasons

General Linear Model: Difference versus BA, Season

Factor	Type	Levels	Values
BA	fixed	2	A, B
Season(BA)	random	19	2009-10, 1991-92, 1992-93, 1993-94, 1994-95, 1995-96, 1996-97, 1997-98, 1998-99, 1999-00, 2000-01, 2001-02, 2002-03, 2003-04, 2004-05, 2005-06, 2006-07, 2007-08, 2008-09

Analysis of Variance for Difference, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
BA	1	2256.0	2492.9	2492.9	5.65	0.046 x
Season(BA)	17	8407.3	8407.3	494.5	0.75	0.745
Error	110	72515.0	72515.0	659.2		
Total	128	83178.2				

x Not an exact F-test

The significant BA factor found in the 2009–10 wet season analysis from above indicates greater downstream (relative to upstream) egg production compared with previous wet seasons. This result supports those reported above for CUSUM control charting, where sustained higher egg production downstream of the mine in the 2009–10 wet season led to test observations moving outside of the (lower) control limit. Taken together, the CUSUM and ANOVA results indicate the need for an assessment or further investigation of possible ecological significance and causes of the higher downstream egg production (see section 6).

6 Impact assessment

6.1 Background

An impact resulting from a change in composition of creek water (which may be the result of inputs from the mine site) may be inferred from a statistically significant change or trend in the difference values associated with control and exposed sites egg production between any two time periods. The assessment of possible mine-related change and significance of any possible impact is based on comparison of the toxicity monitoring test results with any other environmental information, especially water chemistry and hydrology data.

For routine monitoring, the most useful statistical tests to apply are CUSUM control charting, and the BA factor of the two-factor ANOVA, comparing the current (wet) season's data with data from all previous seasons, as demonstrated in section 5.3 above. Assessment of the environmental performance of the Ranger mine, based upon water quality and toxicity monitoring results, are reported each year in the Supervising Scientist's Annual Report.

At the time of publication of this protocol, and for the first time, a significant difference was observed between egg production results (difference values) arising from a particular wet season – the 2009–10 wet season – and previous seasons' results. This result reflected the consistently greater egg production at the Magela Creek downstream (exposed) site in this wet season (Figure 7B).

It should be noted that a significant deficiency in the simple BACIP design applied to biological response data in the single 'impact' stream of interest (Magela) is the weakened inference about impacts that may be made. Where significant statistical change is detected, there may be no way to distinguish between mine-related cause and natural cause that may also have occurred at, say, adjacent streams similar in environmental characteristics as the 'exposed' stream but with no mining activity in the catchments. (Sampling multiple control streams with similar environmental characteristics as the exposed stream minimises the risk of incorrectly labelling a coincident natural event as an impact, a design that characterises macroinvertebrate community-based monitoring for Ranger (Supervising Scientist Division 2011b) but which is not possible for (wet season) access and high potential cost reasons to apply to toxicity monitoring – see ANZECC & ARMCANZ (2000), Appendix 4). This weakened inference places an onus on assessing different lines of evidence in a careful and close examination of potential natural and mine-related environmental factors that could be responsible for the observation(s).

6.2 Assessment of impact

In relation to enhancement in egg production at the downstream exposed site, on average, egg numbers at this site are usually slightly greater than that measured at the upstream control site (Figure 7). Most likely, higher downstream egg production may be attributed to the inputs to Magela Creek of billabong-tributary waters (Georgetown and Coonjimba) at two locations. These tributary-ponded waters have higher temperatures, a higher organic carbon content than main-stem creek waters and, for Coonjimba in particular, elevated concentrations of mine solutes (including $MgSO_4$ and Ca) compared to, low solute Magela creek waters). Higher water temperatures enhance reproductive activity in *Amerianna cumingi* (Jones 1992). Further, both the dissolved salts (providing they are not too high in concentration), increased nutrients and natural organic matter would supplement the food supply and in turn, could enhance egg production, of downstream snails.

Water quality differences between the two Magela monitoring sites are also highly affected by creek hydrology. On a falling hydrograph in Magela Creek, previously-ponded waters from billabongs located between the upstream and downstream sites flow out to the creek, accentuating solute and nutrient differences between the sites (higher concentrations are measured at the downstream site, particularly along the west bank).

A discussion of the possible contributors to the 2009–10 wet season results is provided below to provide background context to assist future assessments of potential causes for test response and to guide the types of investigations that may need to be implemented (see also Humphrey et al 2011).

A. **Operator/methodological error**

An audit of protocol procedures used in the 2009–10 wet season was conducted. The operator conducting and supervising the tests has been the same staff member for the past four wet seasons. While refinements (efficiencies) have occurred in the protocol over this period, mainly as a consequence of moving from creekside to in situ testing, these are very minor and are not regarded as sufficient to influence the results in 2009–10. Moreover, exactly the same procedures have been followed for the past two wet seasons, yet the pattern of results for the 2009–10 wet season differs from 2008–09 test results (see Figure 7B).

B. **Possible anomalies observed at the upstream control site**

While the egg number value from the 3rd test conducted in the 2009–10 wet season appears to be unusually low compared to the corresponding downstream value, the same pattern in reproductive response at this site was also observed for the subsequent two tests (4th and 5th tests) (Figure 6B). For the upstream data from the 3rd, 4th and 5th tests, precision amongst the replicates was similar to that observed at all other times, data from the two independent duplicate containers (each holding 8 pairs of snails) were similar, no outliers were evident in the data, and adult snail mortality over the four day exposure period was well within acceptance limits (data not provided in this report). Based on statistical criteria, there was no reason to consider any of the 2009–10 test data from the upstream site anomalous and 'outlying'.

C. **Possible mine-related changes to water quality associated with U or $MgSO_4$**

While higher concentrations of U are observed in Magela Creek downstream of Ranger during the wet season (but well below the current toxicity-based U guideline value), the dominant mine-derived contaminant entering the creek at this time is $MgSO_4$. Concentrations of this contaminant are conveniently measured by the highly correlated variable, electrical conductivity (EC). The question to be posed is, can this input of $MgSO_4$ lead to enhanced snail egg production at the downstream site?

1. Comparison of field responses with laboratory sensitivities of A. cumingi

Over the concentration ranges tested in the laboratory, there is no evidence of *enhancement* of reproductive responses in *A. cumingi* to either Mg (van Dam et al 2010) or U (Hogan et al 2010), particularly at low concentrations. In the case of U, it must be acknowledged that the lower end of the concentration range tested in the laboratory is well above the concentrations of U measured in the creek. However, for Mg there is an overlap in the lower end of the concentration range tested in the laboratory and concentrations that are actually measured in the field.

According to laboratory concentration-response data for U, no effects upon *A. cumingi* could be expected below about 10 µg/L (Hogan et al 2010) yet ambient concentrations of U measured from grab samples in Magela Creek downstream of Ranger during the 2009–10 wet

season did not exceed 0.2 µg/L (Supervising Scientist 2010, Figure 2.3). The near two orders of magnitude difference between measured concentration of U in the creek and that eliciting toxicological response discounts U as contributing to the relatively higher egg production in snails observed in the 2009–10 wet season.

The laboratory concentration-response data for Mg indicate no impairment of egg production by *A. cumingi* would be expected below about 1-2 mg/L (van Dam et al 2010) (taking into account the ameliorative effects of Ca present in mine waste waters) which is equivalent to an EC of about 20-30 µS/cm (Supervising Scientist 2009, Figure 3.3a). The same toxicity data also indicate that no positive effects (ie increased egg production) would be expected due to increases in Mg concentration above the natural background concentrations of Magela Creek.

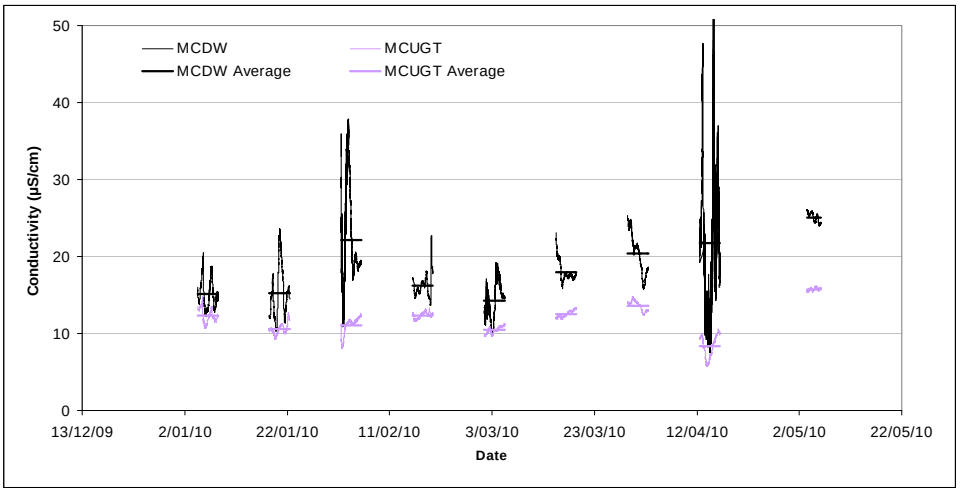


Figure 9 Plots of continuous electrical conductivity (and four-day average values) at monitoring sites in Magela Creek for days in the 2009–10 wet season coinciding with conduct of toxicity monitoring tests

2. Possible gradient in EC observed in previous years between sides of the creek channel at the downstream site

Since the inception of toxicity monitoring in 1992, there have been duplicate containers holding snails at the upstream and downstream sites. In the period from 1991–92 to 2007–08, the duplicate containers at the downstream site either drew water from, or were located on, the east and west sides of the west channel of the creek. From 2009 onward, the duplicate containers have both been located on the west side of the channel only.

Over three wet seasons in the period 2005–06 to 2007–08, EC and other water quality variables were continuously measured at both east and west locations using datasondes. The EC traces from the sondes highlight the EC gradient between the locations. This is caused by incompletely mixed mine waters (with higher EC signature) flowing closely to the west bank of the channel (Figure 10). The continuous readings along the west bank are significantly higher ($P<0.0001$) than corresponding readings taken on the east side of the channel in each of the three years of continuous measurement.

Since 1992 to 2008, the mean egg number representing exposure of snails to both sides of the creek channel has been the same (152 west versus 152 east). For the period 2005–06 to 2007–08 when EC appears to have increased at the downstream site (Supervising Scientist 2010; Figure 2.6), the egg number means have also been similar (128 west versus 131 east, no statistically significant difference). Thus the EC gradient between west and east sides of the creek at the downstream site in this period has not been large enough to result in a statistically different response in snail egg numbers.

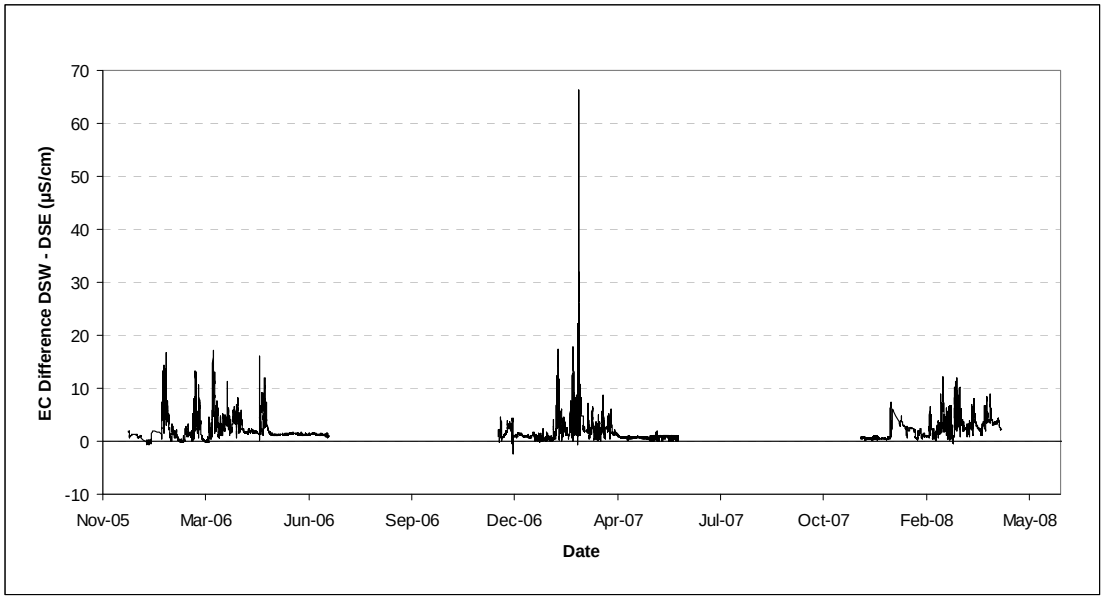


Figure 10 Difference in electrical conductivity (EC) measurements in Magela Creek between west and east sides of the channel downstream of Ranger for the 2005–06, 2006–07 and 2007–08 wet seasons – continuous (hourly) monitoring data (from SSD).

3. EC differences between upstream-downstream sites

Cross-correlation of snail egg production data, both mean egg number per site and upstream-downstream differences, for the nine 2009–10 wet season tests with corresponding continuous water chemistry (including EC) and stream water level data for each four-day period, was conducted using the correlation analysis tool of Excel. The population correlation calculation (also equivalent to Pearson's correlation coefficient) returns the covariance of two data sets divided by the product of their standard deviations.

The four day median value of 10 minute readings of continuous data was used in the analysis, with additional metrics (eg minima, maxima, see Table 2) applied to stream water level data measured close to the downstream site (GS8210009) (Table 2). The reason that the four-day median value was used is that this period corresponds to the same deployment time for the in situ toxicity monitoring method. The results of the analyses are shown in Table 2 (for 7 degrees of freedom (9-2 tests), an r value ≥ 0.666 is significant at $P<0.05$).

(a) *2009–10 wet season*. There was no correlation between any of the EC and egg production measures for the nine tests conducted in the 2009–10 wet season (Table 2; also compare Figures 7B and 9). Indeed, egg numbers actually converged (similar differences) between the sites late in the wet season when greater EC differences were observed (Figure 9).

(b) *EC values observed since 2006.* Related to item 1 above, the incidences and magnitude of 'high' EC events in the 2009–10 wet season appear no greater than for the previous 4 wet seasons for which continuous monitoring data are available (Figure 11), yet egg number differences between upstream and downstream sites are not similarly high in previous recent wet seasons (Figure 7b).

Table 2 Correlations (Pearson *r* values) amongst egg production and continuous water chemistry and stream water level statistical summaries for toxicity monitoring tests conducted in the 2009–10 wet season. Egg number data are means per snail pair from upstream (U), downstream (D) or upstream-downstream differences (Diff), while median data for EC, turbidity and temperature for corresponding sites and four-day test periods were used. Water level (WL) data summarised as maximum, minimum, median, standard deviation, and the maximum fall in water level observed over the four-day period.

	Egg-U	Egg-D	EggDiff
Egg-U	1.000		
Egg-D	0.690	1.000	
EggDiff	0.528	-0.251	1.000
EC-U	0.297	0.248	0.107
EC-D	0.451	0.415	0.116
ECDiff	-0.333	-0.324	-0.066
Turb-U	-0.365	-0.464	0.056
Turb-D	-0.636	-0.638	-0.101
TurbDiff	0.736	0.543	0.347
Temp-U	0.093	0.228	-0.143
Temp-D	0.127	0.257	-0.132
TempDiff	-0.316	-0.307	-0.062
WL-max	-0.527	-0.422	-0.210
WL-min	-0.479	-0.460	-0.101
WL-med	-0.464	-0.506	-0.027
WL-SD	-0.496	-0.387	-0.208
Max fall	-0.483	-0.263	-0.337

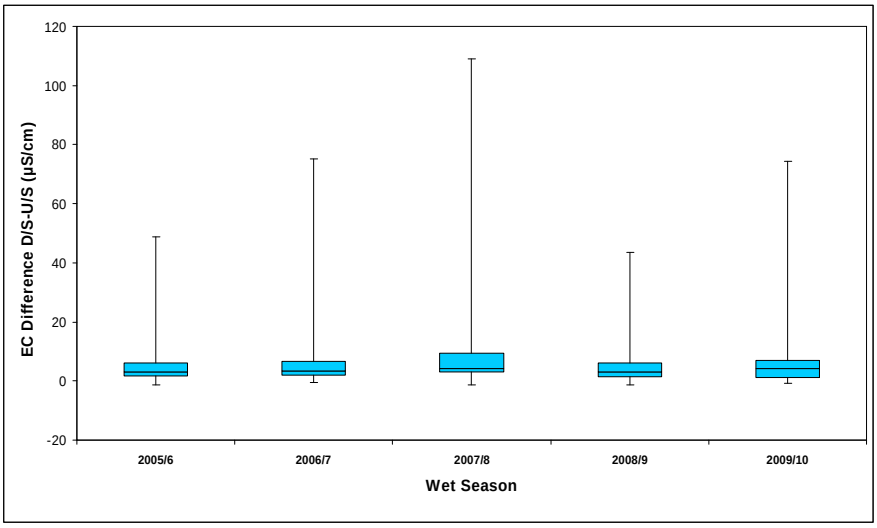


Figure 11 Box plots (median and quartiles) of Electrical conductivity (EC) differences (D/S-U/S) for continuous monitoring sites in Magela Creek

Taken together, the above results indicate that $MgSO_4$ is unlikely to be a significant contributor to the greater snail egg number differences observed in the 2009–10 wet season.

D. Other possible mine- or non-mine-related explanations for the 2009–2010 wet season observations

Water temperature, turbidity, organic carbon and stream flow dynamics are other factors that could potentially explain the 2010 results.

1. *Water temperature*. Water temperature will vary depending upon water levels, cloud cover, riparian vegetation and period of the wet season. Continuous and spot measurements have

shown that while downstream water temperatures in Magela Creek are slightly higher than upstream, the differences in 2010 were very similar across all tests and comparable to differences measured over the past several wet seasons (data not provided). Further, there was no correlation between any of the water temperature and egg production measures for the nine tests conducted in the 2009–10 wet season (Table 2), indicating that water temperature is not responsible for the significantly greater downstream egg number differences in 2010.

2. *Turbidity*. In 2009–10, the mean downstream turbidity measured over the four-day duration of each of the nine toxicity monitoring tests was generally higher relative to corresponding mean upstream turbidity for the same periods (Figure 12).

However, and as shown for EC above, there was no correlation between any of the turbidity and egg production measures for the nine tests conducted in the 2009–10 wet season (Table 2; compare Figures 7B and 12). Upstream turbidities were 'high' during the 8th test (Figure 6) but this corresponded to close upstream-downstream concordance in egg number. Regional (Alligator Rivers Region) and Australian literature suggests that sustained turbidity (ie for at least several days) greater than 20 NTU is required to adversely affect aquatic biota in inland waters (Buckle et al 2010), values which were exceeded but for very short periods only.

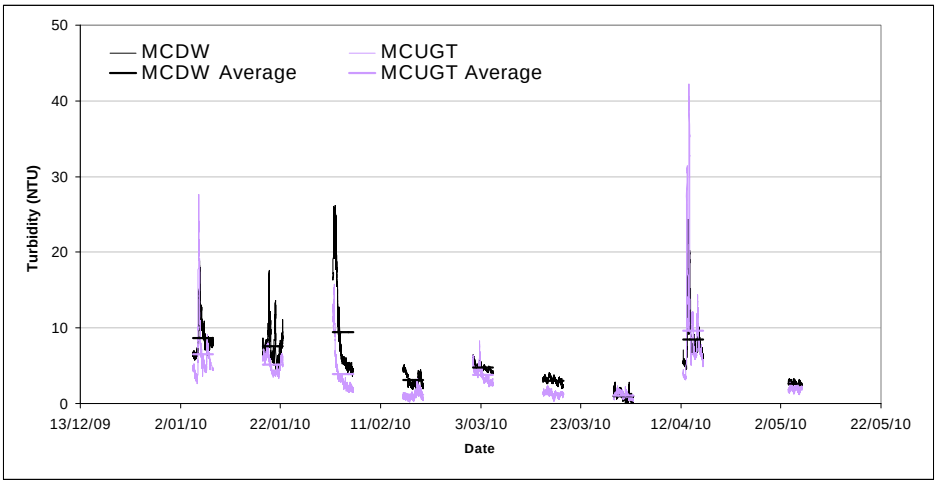


Figure 12. Continuous plots of turbidity (and four-day average values) at monitoring sites in Magela Creek for days in the 2009-10 wet season coinciding with conduct of toxicity monitoring tests. (Note that turbidity traces for test periods 2 and 3 include some error and may be revised in future as ongoing QA/QC are applied to the associated data.)

3. *Organic carbon*. This variable has been measured sporadically over the period of toxicity monitoring (since 1992) and collation of the data was not completed at the time this protocol was prepared. Systematic weekly collection of samples for the measurement of total and dissolved organic carbon (TOC/DOC) commenced with the 5th toxicity monitoring test in 2010 (Figure 13).[5] Downstream values are usually higher than upstream. However, for the tests for which data were gathered in 2010, the relative differences between the sites were very small. There was no correlation between any of the total organic carbon and egg production measures for the five tests of common data conducted in the 2009–10 wet season ($P>0.05$).

[5] Spot weekly collection and analysis of water samples for TOC/DOC discontinued in the 2010–11 wet season at which time a method for collecting and measuring organic material settling in snail containers over the four-day duration of each test commenced (see section 3.5.4).

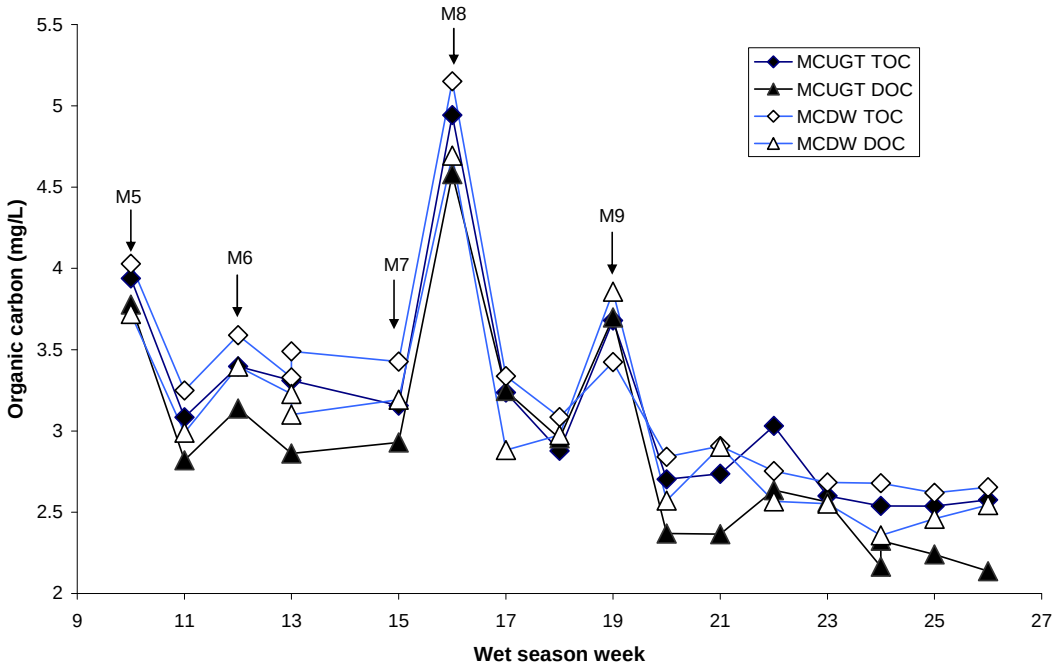


Figure 13 Plots of weekly wet season Total (TOC) and Dissolved (DOC) Organic Carbon in Magela Creek surface waters from grab samples collected from upstream (MCUGT) and downstream (MCDW) sites. Symbols M5 to M9 refer to toxicity monitoring tests 5 to 9 respectively.

4. *Flow dynamics at the downstream site*. As noted above, the greatest influence on water quality at the downstream monitoring site is water draining from billabongs (Georgetown and Coonjimba). A number of water quality variables, including solute concentrations, are enhanced in billabong waters. These inputs could have the potential to enhance snail egg production but, as discussed above, the concentrations of major ion solutes, are unlikely to be the cause. The correspondence of toxicity monitoring tests conducted in 2010 with falling stage in Magela Creek was examined. Water levels measured during the toxicity monitoring tests are shown in Figure 14. The falling hydrograph during the third and eighth tests coincided with peaks in downstream EC, as water initially held back in Coonjimba Billabong flowed into Magela Creek (see Figure 9). No correlation was found between any of the water level data and egg production for the nine tests conducted in the 2009–10 wet season (Table 2; compare Figures 7B and 14).

Monitoring staff have noted, in particular, the deepening of the channel at the downstream monitoring site. This deepening would result in a reduction in water velocity across the stream profile at this location, and hence increase potential for settling of the suspended particulate material. Indeed, increased accumulation of organic material, a potential food source for snails, in the toxicity monitoring containers at the downstream site was noted in the 2009–10 wet season compared with previous years. However, no quantitative measurements of the amount of this settled material were made. Whether or not the increase in settled material inside the test containers at the downstream site was associated with, or accentuated by, a possible increase in erosion rates near the minesite as a result of mine exploration activities in recent years remains to be assessed.

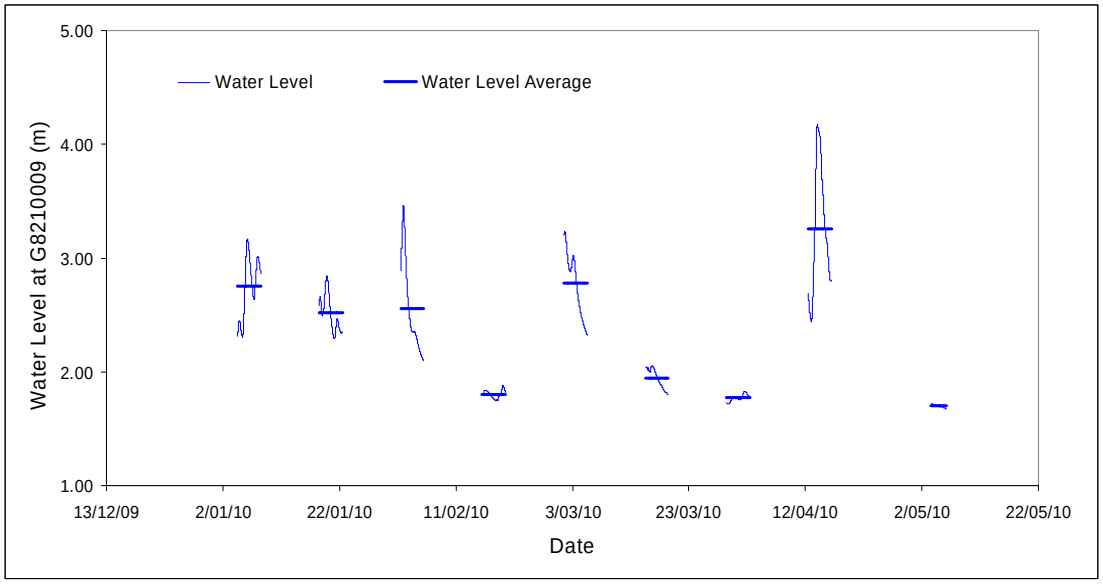


Figure 14 Plots of continuous water levels at Magela Creek downstream (MG009) for days in the 2009–10 wet season coinciding with conduct of toxicity monitoring tests

Summary and further work

A summary of the conclusions from each of above lines of investigation to determine the possible causative factor(s) for enhanced downstream egg production is presented in Table 3. The main mine contaminants, U and Mg, are discounted as contributing to the 2009–10 observations. Altered flow regime at the downstream site resulting in an increase in settled organic material inside the test containers at this site is a more likely explanation.

There are limitations to observational and correlational approaches to drawing inference because of the potentially concurrent mine- and non-mining-related factors that could contribute. Laboratory studies to examine the responses of freshwater snails to a limited matrix of water quality variables, including Mg and organic carbon at low concentrations, would be one path to addressing this issue, albeit resource-intensive and with no certainty that subtle responses in this range of concentrations could be discerned within the limits of precision of the snail test method. Initially it is proposed to implement in 2010–11, a method to quantify the amount (and carbon content) of particulate matter deposited in the in situ test containers, and to assess if there is any positive correlation between the amount (and nature) of deposited material and snail egg production. Whilst spot measurements of dissolved organic carbon have been made in the past, these data do not address the issue of potential food supply and the quantity available over the four-day test period.

There are limitations to observational and correlational approaches to drawing inference because of the concurrent mine and non-mine related factors that could potentially contribute. Laboratory studies to examine the responses of freshwater snails to a limited matrix of water quality variables, including Mg and organic carbon at low concentrations, would be one path to addressing this issue, albeit resource-intensive and with no certainty that subtle responses in this range of concentrations could be discerned within the limits of precision of the snail test method. In the 2010–11 wet season, a method to quantify the amount (and carbon content) of particulate matter deposited in the in situ test containers was initiated (see section 3.5.4). These data will be used to assess if there is any positive correlation between the amount (and nature) of deposited material and snail egg production. Though spot measurements of dissolved organic

carbon have been made in the past, these data do not address the issue of potential food supply and the quantity available over the four-day test period.

Table 3 Multiple lines of evidence to infer the possible cause of relatively higher snail egg production at the downstream site in 2009–10 wet season

Potential causative factor	Potential contributor to enhanced downstream egg production?	
Operator/methodological error	No	• Careful audit of protocol procedures discounted operator errors
Possible anomalies observed at the upstream control site	No	• Comparable precision amongst replicates, as for previous years • Water quality at upstream site not greatly different from that observed in previous years
Uranium	No	• Concentrations measured in Magela Creek well below toxicological thresholds • Enhanced egg production responses at low U concentrations not observed in ecotoxicological studies
Magnesium	Unlikely	• Concentrations measured in Magela Creek at or above toxicological thresholds for short periods but enhanced egg production responses at these (low) Mg concentrations not observed in ecotoxicological studies • Significant cross-channel gradient in Mg concentration at the downstream site not reflected in similar gradient in biological response • No correlation between EC and egg production measures for the nine tests conducted in the 2009–10 wet season • Similar incidences and magnitude of 'high' EC events in previous wet seasons yet pattern of egg number differences between upstream and downstream sites unique to 2009–10 wet season
Water temperature	Unlikely	• Consistent between-site temperature differences amongst nine tests conducted in the 2009–10 wet season • No correlation between any of the water temperature and egg production measures for the nine tests
Turbidity	Unlikely	• No correlation between any of the turbidity and egg production measures for the nine tests conducted in the 2009–10 wet season
Total organic carbon	Unlikely	• No correlation between total organic carbon and egg production measures for the five tests of common data conducted in the 2009–10 wet season
Alteration to flow dynamics at the downstream site	Possible	• Deepening channel at downstream site may have contributed more settled organic material, and hence available food, for snails

Analysis of biological, water chemistry and creek hydrology data will continue to better determine the water quality constituents contributing to enhanced snail egg production downstream of Ranger, and the extent to which mine inputs and other mine-related alterations to water quality and hydrology of the receiving-water billabongs could be contributing.

Should enhancement in snail egg production be linked to stimulatory mine-related effects, further discussion and consideration would be required to determine whether this in fact constitutes an adverse ecological effect.

7 Reporting

Different reporting mechanisms are required for different forums and stakeholder groups. There is a (more or less) chronological sequence of corporate and other reporting from the commencement of the calendar year, as follows:

- Internet
- Reporting to Traditional Owners
- Supervising Scientist Annual Report
- Alligator Rivers Region Technical Committee (ARRTC) and Annual Research Summary (Supervising Scientist Report)
- Additional summary reports for stakeholders

7.1 Internet

As data are acquired during the wet season, plots of the accruing results (per Figure 6) are posted to the SSD website:

http://www.environment.gov.au/ssd/monitoring/magela-bio.html

A short Explanatory Note on any trends or unexpected results accompanies the data.

The additional reports listed above – Supervising Scientist Annual Report, ARRTC papers, Annual Research Summary, any illustrated report for Traditional Owners and other stakeholder report – are also posted to the SSD Website as they become available.

7.2 Reporting results to Traditional Owners and indigenous residents

There are two components to communicating the monitoring program to indigenous people:

1 Informing people of what tasks are to be undertaken, when, by whom and why; and

2 Providing feedback to people on the results of the work and providing assurance that the environment and their lifestyle have been protected.

Communicating the monitoring program occurs through a variety of mechanisms including:

- Involvement of Aboriginal people in the actual monitoring program, especially through employment.
- Regular updates and reports of monitoring results presented by the Aboriginal Liaison Officer at meetings and associations. Larger meetings or Open days may also be planned for this purpose. Monitoring staff (and more senior Darwin based staff) are available to people (particularly Traditional Owners and Aboriginal residents) to answer questions or provide additional information as requested. Information is provided on what programs are to be undertaken and their timetable. Feedback is also sought on any key questions and needs.
- Illustrated report of monitoring results for Traditional Owners and Aboriginal residents.

7.3 Supervising Scientist Annual Report

This report is tabled in Parliament in the latter part of each year. A summary of toxicity monitoring results (which may be an abbreviated version of the summary reports described in

section 7.4 below), including figures illustrating any highlights, is included in the Annual Report.

7.4 Alligator Rivers Region Technical Committee and Annual Research Summary (Supervising Scientist Report)

A verbal summary of results to date for the wet season is reported to the Alligator Rivers Region Technical Committee (ARRTC) for their meeting convened in the wet season in question (typically February-April period) while a full summary report for the wet season is provided to ARRTC for their late dry season meeting. This latter summary is used as the basis for reporting in the ***eriss*** Annual Research Summary (a Supervising Scientist Report), compiled late in the calendar year, together with results from other stream monitoring programs.

The Annual Research Summary is circulated to a wide audience, including the key stakeholders, Energy Resources Australia, the Northern Lands Council and the NT Department of Regional Development, Primary Industry, Fisheries and Resources. A full list of recipients is available from the SSD Publications Officer. The production of the report features on the *What's New* section of the Supervising Scientist's Web page and is available to the public upon request.

The technical reports from sections 7.3 and 7.4 should follow the outline, or cross-refer to accompanying sections containing the relevant information:

1 Brief description and background of the monitoring program.

2 Details of the just-completed wet season and/or anomalies. This includes water flow timing and period of all waste-water dischares or accidental discharge events, and may include unusual weather or hydrological events, etc.

3 Brief description of methods with referral to protocols. Any variations from the accepted protocols and reasons for the variations should be reported.

4 Current wet season's results and comparisons to past wet seasons' trends and findings. This would include summary statistics for the data collected in the current season, BACIP (ANOVA) analysis of egg production difference values, and the relationship, if any, of these biological data to environmental conditions and variables.

5 Evaluation of results and conclusions on the likelihood of an impact having occurred.

6 Recommendations based on conclusions drawn from the evaluation.

7.5 Summary report for stakeholders

Consistent with the reporting to ARRTC and with similar timing, two reports and presentations are provided each calendar year to the Alligator Rivers Region Advisory Committee (ARRAC), representing a wide range of stakeholders for the ARR (not necessarily with technical backgrounds). The reports contain a summary of major results and conclusions, often in a more plain-english form to those reports described in sections 7.3 and 7.4 above.

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