INTERNAL REPORT

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ERISS Protocol for the creekside monitoring of Magela Creek waters
II. Larval black-striped rainbowfish, ***Melanotaenia nigrans***, Survival test

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The Supervising Scientist for the Alligator Rivers Region has research, supervisory and co-ordination responsibilities related to effects on the environment and uranium mining in the Alligator Rivers Region and research and supervisory responsibilities related to effects on the environment of non-uranium mining in a conservation zone declared within the Region.

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ERISS PROTOCOL FOR THE CREEKSIDE MONITORING OF MAGELA CREEK WATERS

II. LARVAL BLACK-STRIPED RAINBOWFISH, *Melanotaenia nigrans*, SURVIVAL TEST.

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FORWARD

The following protocol is in DRAFT form. It is our intention that the written procedures for conducting the larval black-striped rainbowfish survival test for creekside monitoring be handed over to RUM staff for practical implementation. After feedback from company staff, the protocols will be revised.

Eventually, full application of the protocol and analysis of the data gathered under its procedures will require accompanying creekside monitoring information. In particular, there are two internal reports in preparation describing the creekside monitoring systems on Magela Creek (including routine maintenance) and the data gathered so far upon *M. nigrans*. The paper by Humphrey *et al.* (1995) outlines the rationale for the responses used in creekside monitoring and the design (BACIP) employed.

Reference

Humphrey, C.L., Faith, D.P. & P.L. Dostine (1995). Baseline requirements for assessment of mining impact using biological monitoring. *Aust. J. Ecol.* (in press).

DRAFT ONLY

ERISS PROTOCOL FOR THE CREEKSIDE MONITORING OF MAGELA CREEK WATERS

II. LARVAL BLACK-STRIPED RAINBOWFISH, *Melanotaenia nigrans*, SURVIVAL TEST.

1. Objective

Detection, in the field, of adverse effects of dispersed mine waste-waters upon black-striped rainbowfish (*Melanotaenia nigrans)* larvae over a 4-day test period.

2. Principle of the Test

Groups of ten rainbowfish larvae, 0-1 day old, are exposed to Magela Creek water for four days in tanks receiving a continuous flow of creek waters drawn from one of two sources, ie. upstream of the mine site (control water) and downstream of the mine site (test water), at a creekside laboratory. After four days the number of larvae surviving in each treatment are compared. Where significant *changes* in the differences in survival are found to occur between a time series of results from the two sites this is taken as evidence of an unacceptable concentration of mine waste-waters in the creek water.

3. Test organism

The test animal, *Melanotaenia nigrans,* is abundant in the upper reaches of the lowland and escarpment sections of the Magela Creek system. It has a very patchy distribution in the floodplain section and is not abundant there. The fish used for testing are derived from laboratory stocks of adult fish collected from Magela Creek and Burdulba Creek from 1989 to 1994. Additional fish from the wild have been added to the brood stock each year. Larvae in the range of 0-1 days old after hatching are obtained from laboratory culture. The test animals must be free from overt disease and malformation and swim without impediment.

The continuous flow of creek water through the test aquaria provides a continuous supply of natural food and therefore artificial feeding of the young larvae is not required.

4. Equipment

All materials that come into contact with any liquid to which the animals are exposed should be chemically inert. Any pump used for collection of any water should have inert linings and impeller and all tubing used should be of cured plastic.

(a) Test containers

Six test containers are required for each treatment (water type). These consist of transparent and colourless Perspex vessels, rectangular in plan and cross-section, each capable of supporting 10 fish larvae. (Where organic compounds are of concern, glass containers should be used.) Each test container must contain a volume of test solution sufficient to ensure an exposure equivalent to at least 3 L/g of fish but maintaining conditions in which the turbulence associated with inflowing water does not make it difficult for larvae to hold position. A freeboard of at least 5 cm is required above this volume. By way of example, the preceding specifications can be met in a test container that is slightly flared in cross-section (for stacking) with maximum surface dimensions 360 mm in length x 220 mm in breadth, and height of 150 mm. Under test conditions, this container holds a volume of 8L of test water.

The test containers have an inflow and outflow at opposite ends (Fig. 1). Inflowing water is supplied by 4 mm aquarium airline tubing. The tube distributing incoming water is fixed onto an aquarium clamp fitted to the inside of one end of the test container. The tubing should reach a depth of two-thirds of the water level in the container and be fitted with a plastic T-piece at the end to direct flow to each side. The outflow must be covered by a piece of 700μm nylon mesh to prevent loss of larvae. It is important that the mesh does not entrap sufficient detritus as to block the outflow. A convenient and tested design for the outflow that meets these requirements is as follows: 12 mm diameter drainage hose leading from the container is attached on the inside of the container to a PVC threaded spigot (class 18, 32 mm internal diameter). Over the spigot is placed a 30 mm section of PVC pressure pipe (class 12, 40 mm id). Loss of larvae through the outflow is prevented by the covering of nylon mesh held in place over the spigot and PVC section by another circular sleeve made from a 30 mm section of PVC pressure pipe (class 12, 50 mm id).

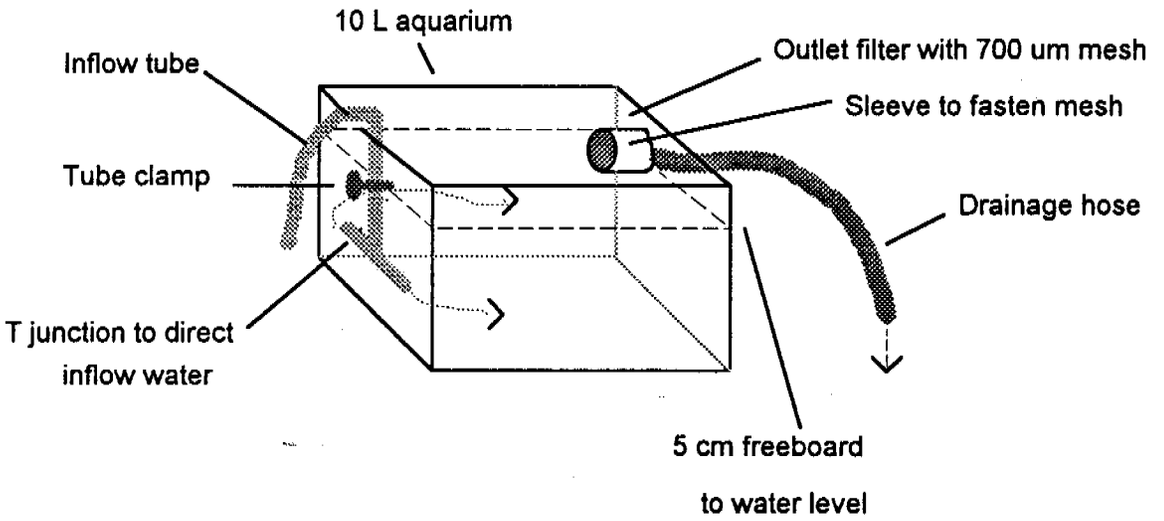

Fig. 1. Diagram of test container for rainbowfish larva survival test

(b) Ancillary

- 25 x 200 mL wide-neck plastic jars with sealable white lids (used for transporting and transferring fish larvae)

- 1 x rectangular plastic container for transporting 20 jars with larvae (e.g. container referred to in (a))

- Max-Min thermometer (one per water treatment)

- Portable dissolved oxygen meter
- Tooth-brush (for cleaning outlets of test aquaria)
- 2 x 60 L plastic bins (for washing equipment at creekside stations)
- 1 x 20 L plastic drum + lid (for transport of control wash water to other sites)
- 2 x 20 L plastic drum + lid (for transport of waste water back to main laboratory)

5. Test Procedure

(a) Production of larvae

The first step in conducting the larval fish survival test is the production of the test larvae. The timing of this process is very important. Egg-substrates must be placed in brood stock aquaria 6 days prior to commencing a test. The Appendix provides a full description of methods for collecting eggs, production of larvae and maintenance of fish stocks.

(b) Test design and experimental lay-out

As stated above, the treatment waters to which fish larvae are exposed are drawn from one of two sources, namely upstream of the mine site (control water) and downstream of the mine site (test water). Two test waters may be drawn: fully-mixed receiving waters (from the station immediately downstream of gauging station GS8210009) or waters from a 'mixing zone' (at present these are located downstream of either the Ranger release pipe or Djalkmara Billabong outflow).

Duplicate waters are drawn independently for each treatment. For control and receiving waters, separate pumps should be located on each side of the creek channel. For the mixing-zone water, pumps should be located 100 m apart on the same side of the creek channel. The duplicate waters of each treatment are pumped to separate header tanks at the respective creekside stations. Each duplicate water then feeds three replicate test containers holding fish larvae (making a total of six replicates for each treatment).

In order to avoid bias in experimental design, test containers are arranged on the laboratory benches so as to achieve a systematic interspersion of treatments. An example of the positioning used at the creekside station where control and mixing zone ('Test') waters are commonly drawn to, is illustrated in Figure 2.

(c) Cleaning of test containers

Prior to initial use in the Wet season, all test containers (and the 200 mL larva transport jars) must be cleaned, using successively, detergent, 10% nitric acid and demineralised water. Prior to use in all tests, containers should be filled with the appropriate waters at the creekside monitoring station and flushed through for a minimum of one hour.

After each test, containers are washed and rinsed with creek water at the creekside station (with no detergent). Any remaining sediment and detritus adhering to containers are wiped off with a towel. This process is much easier if carried out immediately after use while the containers are still wet. After cleaning, the test containers are dipped in 10% nitric acid. The acid wash may be conducted after a test or prior to a new test commencing. If conducted after a test and following the wash, containers are thoroughly rinsed with control creek water that, if necessary, has been transported to

the creekside station for this purpose. If conducted prior to a new test commencing (within 2 hours), containers may be rinsed with treatment water. For either option, the acid-wash solution must be returned to the main laboratory for disposal. Containers left empty between tests are stored upside down on test benches until required.

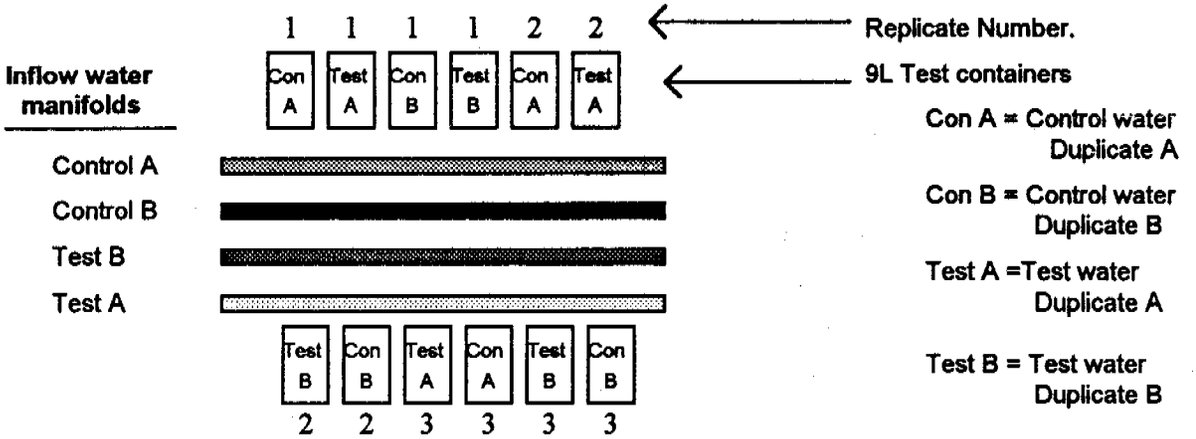

Fig. 2. Diagram showing systematic interspersion of test containers on laboratory bench when two treatments are tested at the one creekside laboratory.

(d) Test responses

Initiating a test. Larvae are procured according to the methods outlined in the Appendix. Each of the treament replicates is allocated 10 fish larvae as follows:

Transfer ten healthy larvae from the hatching bin to one 200 mL plastic jar. After collecting each group of ten larvae, adjust the water level to be about 1 cm from the rim, secure the lid and float the jar in a separate incubation tank to maintain a constant temperature. Repeat until the sixty larvae required for each treatment have been collected. A further 20-30 larvae may be required as replacements for larvae damaged by handling.

Rainbowfish larvae are very small and very susceptible to physical damage arising from transfer between containers. Therefore, extreme care should be exercised in the handling of these animals to minimise mortality. Touching the fish in any way should be avoided and transferring the fish in water from one container to another should be conducted as gently as possible. Larvae tend to swim near the surface of the hatching bins. This enables them to be scooped up with water using an open 200 mL plastic jar. The larvae and water can then be transferred by gently pouring between 200 mL jars. Counting is made easier by placing the white plastic lid under the jar as a contrasting background while viewing the larvae.

Prior to transport to the field, jars containing fish larvae are randomly assigned to one of the treatments. Transport of larvae to each creekside station and their placement in the test containers involves the following sequence of procedures:

1) Jars containing fish larvae are removed from the incubation tank and placed in a plastic container for transport. These are then carefully transported to the creekside station avoiding any excessive agitation of the jars.

2) At the station, a 1/3 water change using appropriate treatment water is undertaken on all jars, taking care not to lose any larvae.

3) One-two cm of water is decanted from each jar and, with the lid removed, the jars from each treatment are then successively placed and floated in individual test containers containing appropriate treatment water.

4) It is essential that the fish larvae are as healthy as possible at the commencement of the test. To ensure this, the larvae are retained in the plastic jars floating in the test containers for a 2-hour period, before they are released into treatment waters. After this period, any larvae that appear to have been injured during counting and transportation are removed and replaced from a supply of excess larvae transported for this purpose. (Two jars with 10 fish should be adequate.) Ten healthy larvae only should remain in each floating jar.

5) Larvae are finally released into their allotted test container by gently submerging each jar and removing it, making sure that no larvae accidentally stick to the surface of jars.

The test is deemed to have commenced after larvae have been released into test containers (step 5 above). This is the time of day that data recordings are made and the time at which a test is terminated on Day 4 of the test. This time should be recorded on data sheets.

Conducting the test. Fish larvae are exposed to the treatment waters for four days. Live fish are counted 24 hours after immersion in treatment water and thereafter at the same time for the following 3 days. On each occasion the number of live fish in each test container is recorded. Counting can generally be done with the naked eye but in poor light conditions the aid of light from a torch may be necessary. The background colour of the bench on which the test containers are placed influences the ease of counting. Some observers prefer a dark background for counting while others find a white background easier. For this purpose, an appropriately coloured background should be inserted under or behind the test container. Counting of fish in a particular container is also made easier by temporarily restricting the inflow line to the container. The fish should be counted a number of times until a consistent number is obtained. After the daily counts, the screens on the container outlets must be cleaned to prevent blockage. The mesh on each outlet is scrubbed with a clean tooth-brush to remove any accumulated sediment or detritus.

On the final day, the number of live fish is obtained by their systematic removal from each test container using a 200 mL clear plastic jar. A tally is made of each fish removed. During this process counting is assisted by placing the white lid of the plastic jar at its base. The larvae are transferred to a collection bucket before continuing the removal and counting of remaining larvae.

Data are entered in a relational database (eg Paradox) that links mortality data with all environmental variables recorded.

6. Test Environment and Conditions

Test environment. The culturing of fish to be used in the tests (Appendix) and all manipulation and testing should be carried out in premises or areas free from harmful vapours and dusts, and undue disturbance. The environment under the creekside shelters should be as uniform as possible, to avoid differences in test conditions amongst the test containers (Brown *et al.*, in prep.). Otherwise, the test containers from within and amongst different water treatments must be arranged under each of the shelters such that there is no bias in test conditions (Humphrey, in prep.). This is achieved by the systematic interspersion described above (section 5 (b)).

Flow rate. Water is continuously pumped from the creek to each header tank in the creekside laboratory using a 12 volt electric pump (Brown *et al.*, in prep.). A 700 µm mesh filter bag is placed on the header tank inlet in order to prevent fish larvae entering the test containers from the creek. Gravity feed, from a single header tank for each treatment duplicate, provides the required continuous flow to each test container. The flow rate to the containers is dictated by both the hydraulic head from the header tank and the numbers of hose lines (connecting test-containers to

supply pipes) concurrently in use (Brown *et al.*, in prep.). Within these limits, when flow is free and unrestricted (i.e. hose lines are not fouled by detritus and algal build-up), the header tanks should deliver water to each test container at a rate of ~300-500 mL/ min. Where this flow condition cannot be met then the flow to each test container should be such as to ensure a replacement not less than 90% over a 2-hour period. It is essential that in any particular test (within and amongst different water treatments) each test container receives the same flow rate, that this rate is as close as possible to optimal (as defined above) and that, as far as possible, amongst different tests a similar flow rate is observed.

Flow rates to each test container must be checked daily at least. Any deviations from the optimal rate should be rectified, e.g. by cleaning or replacing hose lines, fittings etc. or by flushing the entire system (Brown *et al.*, in prep.) and noted on the data records (time, date, degree of flow variation, test containers involved and probable duration). An interruption of continuous flow through a test container for six or more hours would be deemed to be a significant breech of the test protocol and grounds for rejecting the data for affected test containers.

Recording of test conditions. At the commencement of the test and at daily intervals thereafter, water samples are collected from the header tanks containing each treatment duplicate and brought back to the laboratory promptly (within 1 hour) for measurement of pH, conductivity and turbidity. Apart from water samples taken at the commencement of the test, all other samples are collected between 0700 and 0900 hours. Minimum and maximum water temperatures are also read at the time of collecting the water samples, from a thermometer sited in an extra test container for each water treatment, set up without larvae. The minimum and maximum water temperature readings taken each morning relate to the current and previous 24 hour periods respectively. The concentration of dissolved oxygen (DO, mg/L) in the water in each test container is measured *in situ* on the second and fourth days of exposure of fish larvae. (Oxygen concentrations in Magela Creek waters are normally at or above their air-saturation value.) Measurements of DO are made between 0700 and 0900 hours.

It is essential that for proper interpretation of creekside monitoring data, accompanying hydrological and physico-chemical data are available. Apart from measurement of the water quality variables described above, data pertaining to selected metals and major ions will also be required. For creekside monitoring conducted in Magela Creek, collection and analysis of waters for U, Mn, SO_4, Cl and dissolved and total organic carbon of treatment waters should be undertaken at least once during each test.

7. Statistical Analysis of Test Data

Statistical tests for the detection of intermittent (eg. pipeline releases) or sustained and continuous effects arising from dispersion of mine waste waters to creek systems may validly be applied using the BACIP (Before, After Control, Impact, Paired differences) design (Stewart-Oaten *et al.*, 1986, 1992). Survival rate data from a time-series of separate tests are required for this purpose. The statistical procedures to be used are detailed in a separate protocol (Humphrey, in prep.).

For (BACIP) analysis of data derived from trials using rainbowfish larvae it is necessary to tabulate the *mean number of fish surviving after four days in each treatment*. Analyses of the test data are conducted as follows:

Step 1: Rejection of invalid data

Tabulate numbers of dead and surviving fish over the four days in each test container. Any breech in protocol that may have caused a loss of larvae, other than from exposure to treatment waters, can be

considered as rendering the data from a replicate invalid. These data are excluded from further analysis. Such breeches of test protocol may include:

- The over-flowing of test tanks containing larvae (e.g. as a result of blockages in the outflow from tanks). If no loss of larvae is recorded, despite an overflow, the replicate is retained in the analysis.

- Significant interruption to continuous flow through a test container (see above, Section 6, Flow rate).

Step 2: Pooling of replicates

Before calculating an overall mean survival rate for each treatment (by combining results of all replicates), it is necessary to establish that the proportions of dead/ alive fish amongst replicates are not significantly different from one another. The homogeneity of the 6 replicates is checked using the standard chi-square test for the homogeneity of proportions (e.g. Zar, 1984: section 5.8). MINITAB software is convenient for this operation. Data are entered in **two columns** - *'alive'* and *'dead'* - of ***n* replicate rows**. A worked example of the chi-square test, as applied to fish-embryo survival data (OFR 51), is provided in Brown (1992). Data are analysed at the 1% level.

If the chi-square analysis is significant ($P<0.01$) it may indicate the presence of one or two 'offending' replicates. Up to two replicates (from the 6) may be deleted to ensure that further data analysis can proceed. This arbitrary rule (allowing removal of two outliers) is based on practical grounds since the test data are costly to obtain and should not be totally discarded, if possible. Brown (1992) advocated removal of one outlier from 3 replicates in the ERISS fish-embryo gudgeon test on the same grounds. Removal of 2 outliers from 6 replicates, therefore, does not appear unreasonable. If there are 3 or more aberrant replicates, the test is terminated and the experimental procedure reviewed.

Using those replicate data for which homogeneity has been demonstrated, a pooled count of r surviving juveniles from n initial fish larvae is calculated as the survival rate for each treatment.

Note that although the comparison of the control data with other treatments for a single test can be undertaken using a modified Dunnett's test as outlined by Brown (1992), a significant difference between or amongst treatments in a single test does not necessarily infer a mining related cause.

Incompatibility of data between treatment duplicates

Should there be no ready explanation for a significant difference observed between duplicate data from the fish larva survival response, all treatment data are rejected. Should such a significant difference be readily explained by inappropriate experimental procedures applied to one of the duplicates (e.g. pump failure), data from the 'unaffected' duplicate may be used in graphical portrayal of trends in a time series of data but should not be used in formal hypothesis testing.

8. References

Brown, G.H. (1992). Statistical procedures for protocols OFR 51, 56, 57 and 58. Supervising Scientist for the Alligator Rivers Region. Open File Record 97: 38 pp.

Stewart-Oaten A., Murdoch W. & Parker K. (1986). Environmental impact assessment: 'Pseudoreplication' in time? *Ecology* 67, 929-940.

Stewart-Oaten A., Bence J.R. & Osenberg C.W. (1992). Assessing effects of unreplicated perturbations: no simple solutions. *Ecology* 73, 1396-1404.

Zar, J.H. (1984). *Biostatistical Analysis*. 2nd edn, Prentice-Hall, Englewood Cliffs, New Jersey.

Appendix
Fish husbandry and production of larvae

1. Equipment

- Eighteen 50-60 L breeding tanks (glass or plastic aquaria) containing under-gravel filters.

- 2 x aquarium aerators with activated charcoal filters (for incubation containers)

- 2 x aquarium water heaters

- Siphon tube with end covered by 500μm nylon mesh for reducing volume in incubation tanks when collecting larvae)

- Standard thermometer (for checking incubation tank temperatures)

- 20 x egg-laying substrates. These consist of numerous stands of wool about 15 cm long attached to a floating hoop (10 cm diameter) of plastic tubing. The dangling strands of wool apparently simulate vegetation and the fish attach their eggs to these, usually in the early morning.

- 1 x 20 L plastic bucket/water container (for control washing water)

- 1 x 10 L plastic bucket (for carrying fish eggs)

- 2 x 90 L plastic bins (incubation tanks)

2. Brood stock maintenance

Large volume (50-60 L) breeding tanks containing under-gravel filters and creek water obtained from the control creekside monitoring pumps on Magela Creek are used to hold adult fish. Six to eight fish, 2 males and 4-6 females, are placed in each tank. The minimum size of brood-stock fish is approximately 4 cm and care is taken to maintain a similar size range (± 1 cm) in each tank. This is done to reduce damage to fish from agonistic and courtship behaviour that would otherwise occur if there was an imbalance of sizes, and also to match the gamete production of individuals during spawning.

Rainbowfish breed continuously throughout the Wet season (October-May). To maximise egg production over this period, fish are fed twice daily with flake food (e.g. Wardley's tropical) and once a day during weekdays with live decapsulated brine-shrimp.

A 1/3 water change is conducted bi-weekly.

3. Incubation conditions

The incubation period of rainbowfish eggs is 4-5 days at 30°C. Hence, if planning for a test to commence on a Monday it is necessary to begin egg collection on the Tuesday afternoon of the week prior to the test. The incubation period varies with temperature so it is necessary to maintain a constant incubation temperature so that eggs hatch by a specified time. Place two clean 90 L plastic incubation tanks (e.g. Nally bins) in an air-conditioned room at 25°C, and three-quarters fill with fresh creek water. To each incubation tank add a water heater calibrated to 30°C and an aerator with an activated carbon air-filter. Place lids on the tanks to allow incubation temperature to be reached. (Note that power failures can cause problems with incubation time under these conditions.)

4. Collection of eggs

For a test commencing on the following Monday, place one woollen egg-laying substrate in each breeding tank on the Tuesday afternoon at least two hours *after* fish have been fed. This is necessary to avoid the contamination of egg substrates by food that might otherwise result in increased incidences of fungal infections.

On the morning of the following day (Wednesday) remove egg substrates from breeding tanks before feeding fish (to avoid contamination), place these in a bucket of control creek water and, when all are collected, transfer to the incubation tanks that were prepared on the previous day. The incubation tanks should be covered and kept out of direct sunlight.

5. Incubation of eggs and production of larvae

The general procedure for obtaining rainbowfish eggs is outlined by Holdway *et al.* (1988). All waters used in the egg production process should be control creek water and all materials used should be thoroughly cleaned and rinsed before use to reduce the risk of transfer of pathogens.

6. Handling of larvae

Because the larvae are utilised in the test procedure within 24 hours of hatching, they do not require articial feeding. When required, the larvae are collected and transferred to test chambers according to procedures outlined in the protocol.

Although it is inevitable that there is some temporal fluctuation in abundance of natural food received by the larvae in the test containers located at the creekside stations, there is good survival and detectable growth of larvae under the full spectrum of flow conditions occurring in the creek between January and April, suggesting that the food supply is adequate to maintain healthy fish.

7. Larva production capacity

It is obviously very important to have sufficient larvae to conduct the test as designed. The number of brood stock maintained must be more than adequate to supply larvae for the largest test design envisaged. Whilst the fish can spawn every day during the Wet season, the number of eggs produced varies considerably among days. Consequently, for reliable egg production many breeding groups may be required. As a guide, at least 6 such groups provided 150-200 0-2 day-old larvae at 4-day intervals during the 1990-91 Wet season, by collecting eggs on two successive days (rather than on a single day as outlined above, 4. Collection of eggs). In the 1993 Wet season, 18 such breeding tanks provided a reliable source of larvae at a narrower age range (0-1 days old) by collecting eggs on a single day and this practice has continued.

If problems do arise with egg production (e.g. frequent power failures), the strategy of collecting eggs on two successive days may provide a short-term solution.

References

Holdway, D.A., M.M. Wiecek, & V.M. Brown (1988). OSS test protocols for the biological testing of waste waters for release into Magela Creek. III. Larval Rainbow-fish test (*Melanotaenia splendida*). Supervising Scientist for the Alligator Rivers Region. Open File Record 53.