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Assessment of the suitability of the black-striped rainbowfish (*Melanotaenia nigrans*) as a laboratory toxicity testing organism

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Summary

The suitability of the black-striped rainbowfish, *Melanotaenia nigrans*, as a laboratory organism for toxicity testing was investigated, primarily as a tool for assessing mining wastewaters and developing water quality guidelines in the wet-dry tropics of northern Australia. *M. nigrans* was found to be a prolific and reliable breeder if fed a varied but specific diet, and if well maintained in clean aquaria. In addition, survival of *M. nigrans* larvae over 96 h was high when the brood stock were maintained in good condition. Test water water type (artificial or natural) and volume did not appear to affect larval survival over 96 h. When larval survival was poor, the addition of the exogenous food source, Aquasonic® freshwater fry starter (one of three food types tested), greatly enhanced the survival of larvae, although the ration administered (ie 0.5, 1 or 2 µl/ml) had no effect. Having demonstrated adequate survival of larval *M. nigrans*, the acute toxicity of copper (Cu), and the effect of feeding on Cu toxicity were assessed. Both Cu and feeding had a significant effect on mean survival of *M. nigrans* larvae. In the presence of a food source (Aquasonic®), Cu had no effect on larval survival up to the highest concentration tested, of 100 µg/L. In the absence of a food source the survival of larvae decreased with increasing Cu concentration, with the effect being significant at 100 µg/L.

M. nigrans has the potential to be a suitable laboratory toxicity testing organism, due to the successful survival of larvae, unfed, over a 96 h test period. A comparison of the senstivity of *M. nigrans* and the currently used fish species, *Mogurnda mogurnda*, to toxicants relevant to northern Australia is required before adopting *M. nigrans* as a standard laboratory toxicity testing organism. However, it is recommended that a laboratory acute toxicity test using larval *M. nigrans* be adopted for use in the Alligator Rivers Region, to complement the established *in situ* (creekside) toxicity test using the same species.

Contents

1. Introduction

Toxicity bioassays using fish, particularly their early life stages, or larvae, have become important tools for assessing the quality of natural waters or the effects of known environmental pollutants. The standard fish acute toxicity bioassay involves exposing newly-hatched larvae (usually < 24-h-old) to a pollutant or sample of a water body, and monitoring their survival over 96 h (4 days) (Sprague 1971). The other major characteristic of such tests is that the larvae are not provided with an exogenous food supply before, or during the bioassay. As a result, when selecting appropriate species for toxicity testing purposes, the ability to survive for 96 h on the nutrients supplied in the yolk-sac is highly important. Feeding has generally not been incorporated into fish acute toxicity tests as it can result in interactions between the food and toxicant, greatly complicating toxicity. This is a particularly undesirable situation when using toxicity bioassays to derive water quality guidelines for chemicals.

Within the Alligator Rivers Region (ARR) in the Northern Territory of Australia, eight local fish species were previously assessed for their suitability as laboratory toxicity testing organisms, for use in monitoring the toxicity of mine wastewaters from Ranger uranium mine (Holdway et al 1988). The black-striped rainbowfish (*Melanotaenia nigrans*) was among the fish species assessed, and while being considered suitable for hatchability, older larval survival and adult reproduction tests, was not recommended for early larval survival studies (Holdway et al 1988). This limitation was attributed to the difficulty of successfully rearing larvae in the first week after hatching (post-hatch), and as a result, it was recommended that more basic research into the biology and rearing of the fish was required before the species could be used for acute larval survival bioassays (Holdway et al 1988). Consequently, a standard laboratory acute toxicity test was developed using another local fish species, the purple-spotted gudgeon (*Mogurnda mogurnda*), the newly hatched larvae of which showed excellent survival over 96 h (Hyne et al 1996).

In contrast to the above findings for laboratory studies, an assessment of the suitability of different fish species for *in situ* or 'creekside' toxicity tests within the ARR found newly-hatched *M. nigrans* to be a suitable test organism (Humphrey and Brown 1991). Their consistent survival over 96 h was largely attributed to a natural source of food in the incoming water. Subsequently, a standard test was developed for the *in situ* assessment of mine wastewater releases into freshwater systems using *M. nigrans*. However, the use of two different fish species for laboratory and *in situ* toxicity testing is an undesirable situation, as it does not allow for field validation of laboratory results, or vice versa. In addition, laboratory toxicity bioassays carried out on pre-release wastewaters using *M. mogurnda* are in no way comparable to *in situ* toxicity bioassays carried out on creek water contaminated with the same wastewater, using *M. nigrans*.

The current *M. mogurnda* laboratory toxicity bioassay has advantages in that control larvae consistently survive unfed for 96 h (4 days), resulting in very few invalid tests. However, there is a major limitation associated with the culturing and use of this species, further justifying the consideration of other fish species. *M. mogurnda* breed in pairs, with the male fish guarding and cleaning the egg mass during the period of embryonic development. As development lasts approximately four days, breeding pairs can only produce a maximum of one batch of eggs every four to five days, making the availability of larvae for toxicity tests

potentially difficult. In addition, spawning activity decreases markedly when ambient water temperatures increase much above 30°C (C. Camilleri pers. obs.), as is the case during October to December in ***eriss***' outdoor aquaculture facility.

In contrast, there are no such limitations associated with *M. nigrans*. Adult fish breed in groups, producing large batches of eggs on a daily basis, particularly at warmer temperatures. Another advantage of using *M. nigrans* is that several rainbowfish species are commonly used throughout Australia as standard toxicity testing organisms (eg *M. splendida inornata*, *M. duboulayi*, *M. fluviatilis*; Bywater et al 1991; Sunderam et al 1992; Reid et al 1995; Holdway 1996). In addition, if a laboratory test using *M. nigrans* can be developed, their current use for *in situ* toxicity assessments in the ARR will allow for direct comparison of field and laboratory data.

Aims

The initial aim of the current project was:

to establish successful breeding groups of *M. nigrans* in the laboratory, and to determine whether sufficient numbers of eggs for toxicity bioassay purposes can be produced on a regular basis.

If successful culturing was demonstrated, three further aims of the project were:

i) to investigate the survival of unfed *M. nigrans* larvae in a variety of water types (natural and artificial);

ii) to investigate the use of an exogenous food source if adequate survival could not be demonstrated, and;

iii) to assess the effects of the reference toxicant, copper, on the survival of *M. nigrans* larvae, assuming adequate control survival can be demonstrated.

2. Materials and Methods

2.1 Culturing techniques and egg collection

For details on the establishment of breeding aquaria and the appropriate collection and incubation of eggs, refer to Appendix A. Briefly, eggs were collected using woollen 'mop' artificial spawning substrates placed in the breeding aquaria overnight, for approximately 15 h. The mops were removed from the breeding aquaria and placed in 70 L incubation tanks maintained at 27°C. The eggs were incubated in the tanks for 4 - 6 days until hatching. Upon hatching, larvae were collected gently from the incubation tank with a clean plastic beaker and placed in a container for transferral to the testing laboratory, and for immediate use in an experiment.

2.2 Experiment 1: Survival of larval *M. nigrans* in different water types

This experiment assessed the effects of different water types, including two artificial and two natural media, on the survival of *M. nigrans* under laboratory toxicity bioassay conditions. The treatments were as follows:

i) ***eriss*** (Magela Creek) synthetic water (pH 6.0)

ii) ASTM reconstituted freshwater (pH 6.5)

iii) Buffalo Billabong water: Filtered

iv) Buffalo Billabong water: Unfiltered

Each treatment consisted of three replicates, each with 10 rainbowfish larvae per replicate. Unfiltered Buffalo Billabong water was considered to be a positive control, as *M. nigrans* larvae have been shown to survive for 96 h in *in situ* toxicity bioassays in unfiltered Magela Creek water (Humphrey and Brown, 1991)[1]. ***eriss*** synthetic water (see Appendix B) has been developed to simulate natural Magela Creek water excluding the organic content, and is currently used in standard toxicity tests at ***eriss***. ASTM reconstituted freshwater (ASTM 1992; see Appendix B) is a broadly applicable medium, deficient in any trace metals and organic constituents, for which the hardness and pH can be manipulated to represent the water body of interest. The ASTM water was trialed as a potential substitute for the highly specific ***eriss*** synthetic water, as research broadens to focus on regions other than the Magela Creek catchment.

The experimental conditions and test procedure were as per the established protocol for *M. mogurnda* (BTT-E) approved by the NTU AEEC (Ref. No. 97016). For details refer to Markich & Camilleri (1997).

2.3 Effects of food type and ration, and test water volume on the survival of larval *M. nigrans*

The established protocol for the *M. mogurnda* toxicity test stipulates 30 ml as the volume of test water per replicate. A larger volume of water was trialed to establish that 30 ml is an adequate volume. Due to the reduced yolk sac of newly-hatched rainbowfish larvae survival over 96 h may be unsatisfactory to carry out 96 h toxicity tests. The addition of a food source may be necessary for 96 h survival of the rainbowfish larvae. Two experiments were carried out, as described below.

2.3.1 Experiment 2a: Effect of test water volume and feeding

This was a 'screening' experiment that assessed the effects of an increased test water volume, and a food source on the survival of newly-hatched rainbowfish larvae over 96 h. Treatments were as follows:

i) 30 mL ASTM reconstituted water (control)

ii) 150 mL ASTM reconstituted freshwater

iii) 30 mL ASTM reconstituted water + 60µL of Aquasonic® "Freshwater" Fry Starter fish food

Each treatment comprised three replicates, each containing 10 rainbowfish larvae. The experimental conditions and test procedure were as per the established protocol referred to in section 2.2

2.3.2 Experiment 2b: Effect of food type and ration

This was a more comprehensive experiment that assessed the effects of three different types

[1] Buffalo Billabong is within the Magela Creek catchment.

and three different rations of food on the survival of newly-hatched rainbowfish larvae. The food types were as follows:

i) Axenic culture of unicellular algae *Chlorella* sp. (1×10^6 cells/ml)

ii) Wardley's Liquid Small Fry® Baby Fish Food (egglayer formula).

iii) Aquasonic® "Freshwater" Fry Starter

Each food type was tested at three levels: 15µl, 30µl and 60µl (or 0.5, 1 and 2 µl/ml). This resulted in a total of 9 treatments, each with three replicates containing 6-10 rainbowfish larvae. A no-food treatment was also run con-currently, but was not part of the experimental design. For details on the Wardley's® and Aquasonic® food types, refer to Appendix C. The experimental conditions and test procedure were as per the established protocol referred to in section 2.2.

2.4 Experiment 3: Acute toxicity of copper and the effect of feeding on copper toxicity to larval *M. nigrans*

Newly-hatched *M. nigrans* larvae were exposed to six concentrations of copper ($CuSO_4.5H_2O$); 0, 1.0, 3.2, 10, 32 and 100 µg/L. The effect of each of these concentrations was also assessed with and without a food source; 0 or 15µl (0.5 µl/ml) of Aquasonic® fry food per day. The type and ration of food was determined from the results of experiment 2b. The experimental design totalled 12 treatments, each with three replicates, containing 10 rainbowfish larvae per replicate. The experimental conditions and test procedure were as per the established protocol referred to in section 2.2.

2.5 Statistical analysis

All percent survival data were arcsine transformed to ensure they were normally distributed, as follows:

$$x_i = \left(\arcsin \sqrt{\frac{x}{100}} \right)$$

where x is the percent survival for a particular replicate (Zar 1984):

Data were also analysed for heteroscedasticity by way of the chi-square test for the homogeneity of proportions (e.g. Brown 1992).

Experiments 1 and 2a were analysed by 1-way fixed-effects analysis of variance (ANOVA), with water type and treatment being the factor of interest, respectively. Experiments 2b and 3 were analysed by 2-way fixed-effects ANOVA, with food type and food level, and Cu concentration and food level being the factors of interest, respectively. Effects were considered significantly different if $P \leq 0.05$. Differences among treatment means were located using Tukey's Honestly Significant Difference (HSD) Test, again at $P \leq 0.05$.

3. Results

3.1 Experiment 1: Survival of larval *M. nigrans* in different water types

Mean percent survival of *M. nigrans* larvae over 96 h was not affected by the type of water in

which they were kept for the 96 hour test (Table 1; $P > 0.05$, F-crit 4.066). As can be seen in Table 1, survival after 96 h was poor, varying between 40 to 60%. Figure 1 shows the pattern of mortality over the 96 h period. Larval survival was high following the first 48 h, but had decreased considerably after 72 h. Although water type had no significant effect on larval survival, ASTM water was chosen for the subsequent feeding and toxicity tests, due to marginally better survival of fish, and the broader applicability of the medium.

Table 1: *Experiment 1.* Mean ± SE percent survival of newly-hatched *M. nigrans* larvae in four different water types after 96 h ($n = 3$).

	eriss synthetic water	ASTM reconstituted water	Unfiltered BB* water	Filtered BB water
Percent survival	40.00 ± 15.27	56.70 ± 14.53	40.00 ± 11.54	50.00 ± 15.27

* BB: Buffalo Billabong

NB - There were no significant differences between treatments.

3.2 Effects of food type and ration, and test water volume on the survival of larval *M. nigrans*.

3.2.1 Experiment 2a: Effect of test water volume and feeding

The volume of test water had no effect on the mean percent survival of rainbowfish larvae over 96 h ($P > 0.05$, F-crit 2.776). In addition, there was no significant difference in the mean survival of larvae over 96 h with the presence or absence of 60µL of Aquasonic® fry food ($P > 0.05$, F-crit 2.776). Interestingly, larval survival after 48 h was almost two times greater in all three treatments than in experiment 1. The potential reasons for this are discussed in section 4.

Table 2: *Experiment 2a.* Mean ± S.E. percent survival of newly-hatched *M. nigrans* larvae in two volumes of ASTM water and with the presence or absence of food in 30mL of ASTM water after 96 h ($n = 3$).

	30mL ASTM water	150mL ASTM reconstituted water	30mL ASTM water + 60µL Aquasonic® food
Percent survival	90.00 ± 5.77	80.00 ± 5.77	76.66 ± 12.01

NB - There were no significant differences between treatments.

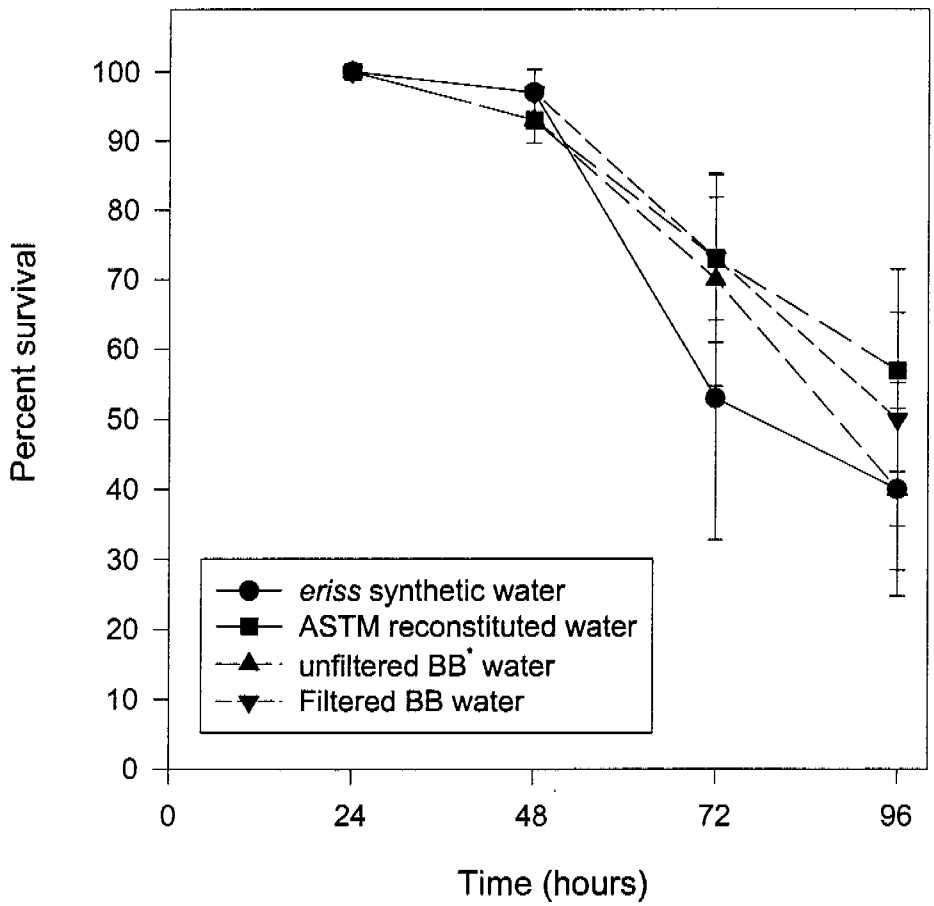

Figure 1: *Experiment* 1. Mean (± SE) percent survival of newly-hatched *M. nigrans* over 96 h in four different water types (n = 3).

* BB: Buffalo Billabong water.

NB - Larvae were not fed during the experiment.

3.2.2 Experiment 2b: Effect of food type and ration

Food type had a significant effect on larval survival, whereby Aquasonic® fry food resulted in a greater mean percent survival than the other two food types (Table 3; $P < 0.05$, F-crit 14.163). Food level (ie 15, 30 or 60 µL) had no effect on the mean survival of larvae, even for Aquasonic® fry food (Table 3; $P > 0.05$, F-crit 1.167). Finally, there was no interaction between food type and level ($P > 0.05$, F-crit 2.151). Except for the Aquasonic® fry food treatments, larval survival was markedly lower than in experiment 2a (section 3.2.1), more closely resembling the results of experiment 1 (section 3.1). Similarly, the no-food treatment (in ASTM water) carried out con-currently to the feeding experiment resulted in a larval survival of only 42.33 ± 11.35% (mean ± SE) after 96 h. Again, potential reasons for this are discussed in section 4.

Table 3: *Experiment 2b.* Mean ± S.E. percent survival of newly-hatched *M. nigrans* larvae fed with different food types at different levels, after 96 h ($n = 3$).

	Food type		
Food level	Algae	Wardley's® fry food	Aquasonic® fry food
15µL	36 ± 6.66^a	63 ± 14.52^a	93 ± 3.33^b
30µL	50 ± 9.81^a	23 ± 18.55^a	90 ± 4.66^b
60µL	50 ± 9.81^a	36 ± 8.82^a	95 ± 4.66^b

Values that share the same superscript letter are not significantly different ($P > 0.05$).

3.3 Experiment 3: Acute toxicity of copper and the effect of feeding on copper toxicity to larval *M. nigrans*

Both Cu and feeding had a significant effect on the mean survival of larvae (Table 4; $P < 0.05$, F-crit 3.439 and 38.239, respectively). However, there was also a significant interaction between copper and feeding on the mean survival of larvae ($P < 0.05$, F-crit 3.744), whereby larvae unfed during the exposure period were more sensitive to copper. Percent survival of unfed larvae exposed to 100 µg/L Cu was significantly lower than unfed control larvae (Table 4; $P \leq 0.05$), while Cu had no effect on the percent survival of fed larvae (Table 4; $P > 0.05$). While percent survival of unfed larvae exposed to 32 µg/L Cu also appeared to be reduced compared to controls, and even fed larvae exposed to 32 µg/L Cu, the effect was not statistically significant (ie $P = 0.063$). The decrease in sensitivity of larval rainbowfish to Cu when fed with Aquasonic® fry food compared to unfed larvae is also presented graphically in Figure 2.

As with experiment 2a, control larval survival after 96 h was between 90 - 100%, regardless of whether the lavae were fed or unfed.

Table 4: *Experiment 3.* Mean ± SE percent survival of newly-hatched *M. nigrans* larvae exposed to copper and either fed or not fed 'Aquasonic' fry food ($n = 3$).

	Copper (µg/L)*					
Feeding	0	1	3.2	10	32	100
Unfed	90 ± 1.0^{a}	$83 \pm 0.88^{a,b}$	93 ± 0.33^{a}	$87 \pm 0.33^{a,b}$	$77 \pm 0.66^{a,b}$	43 ± 0.88^{b}
Fed**	100 ± 0^{a}	97 ± 0.33^{a}	100 ± 0^{a}	98 ± 0.33^{a}	100 ± 0^{a}	100 ± 0^{a}

Values that share the same superscript letter are not significantly different.
* Copper concentrations are nominal.
** Food was provided in the form of 15 µL Aquasonic® fry food per day.

4. Discussion

Survival of control larvae in standard toxicity tests must be above 80% for the test to be considered valid (Hyne et al 1996). Experiment 1, which assessed the effects of four different water types, resulted in very low mean survival, all being well under 60%. This result was consistent with the initial prediction, based on previous research, that the rainbowfish larvae would not survive for 96 h unfed. It was thought that the larvae should survive in the unfiltered Buffalo Billabong water due to the presence of natural food and organic matter, which in the 'creekside' *in situ* tests appears to be the case (Humphrey & Brown 1991). However, larvae survived no better in natural, unfiltered billabong water than in the two artificial water types, devoid of organic material. The reasons for this were unclear, but may have been due to differences between the two testing situations. During *in situ* testing, the test water is pumped directly from the creek to the nearby test aquaria, while in laboratory toxicity testing, the water must be collected from the source, transported to the laboratory, and in some cases stored for a short period at 4°C. In addition, test animals in the laboratory are kept in a smaller volume of water (ie 10 larvae per 30 ml compared to 10 larvae per 1500 ml), in an incubator under an artificial light source. Such a situation may have proved too stressful to rainbowfish larvae, thus adversely affecting their survival, regardless of the water-type.

The poor survival reported in the first experiment precipitated a small feeding and test volume trial in an attempt to gain some specific information on reasons why the larvae were not surviving for the duration of the test period. It was found that the volume of test water had no significant effect on mean survival of larvae, discounting this as a possible reason. Furthermore, larvae fed with a commercial fry food (Aquasonic®) during the course of the experiment did not exhibit greater survival than unfed larvae. However, in contrast to the first experiment the mean survival of larvae in ASTM water with no food source (ie control larvae) was well above 80%. Therefore, it was demonstrated that newly hatched black-

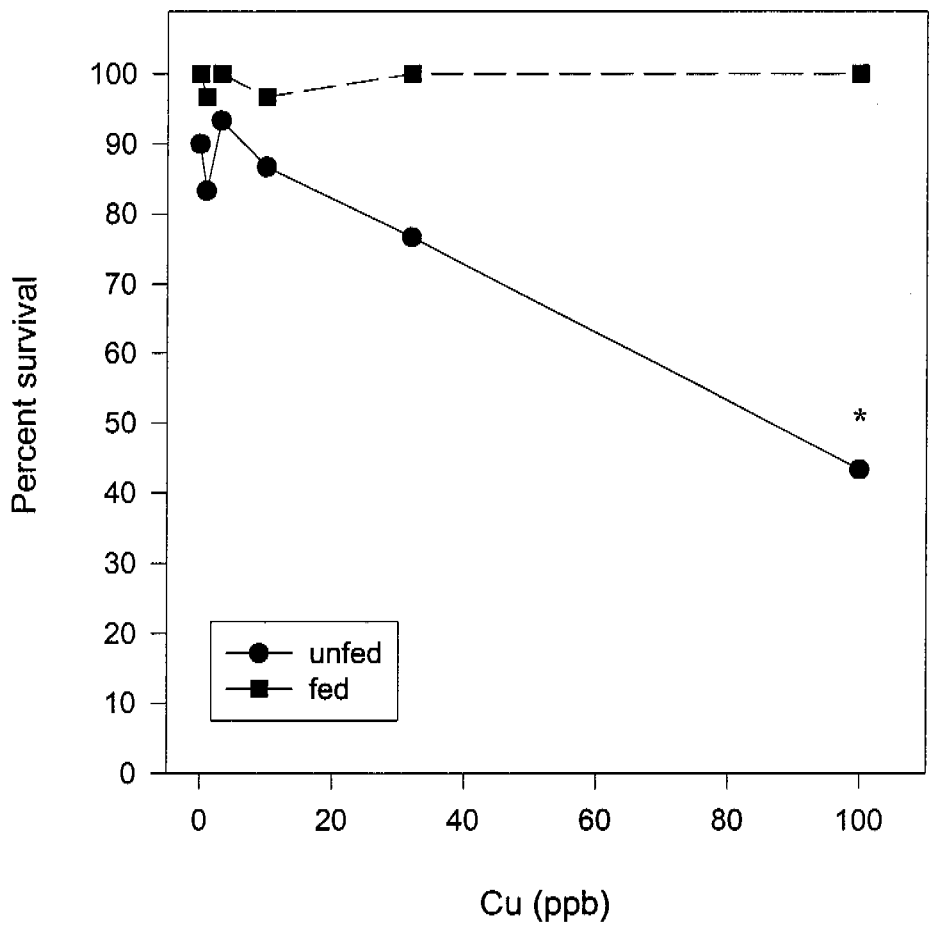

Figure 2: *Experiment 3.* Mean ± SE percent survival of newly-hatched *M. nigrans* larvae exposed to six concentrations of copper, and fed or unfed with 15 µL Aquasonic® fry food per day ($n = 3$).

* indicates a significant difference from controls ($P \leq 0.05$).

striped rainbowfish could in fact survive under laboratory toxicity testing conditions for 96 h, unfed. What required further consideration were the reasons for the apparent differences in survival between experiments, as is discussed below.

Rainbowfish eggs from an overnight spawning period generally hatch over two days, some obviously taking longer than other eggs to develop. The small feeding/volume experiment (experiment 2a) was carried out using larvae that hatched on the first hatching day. In contrast, experiment 2b commenced the following day, using larvae that hatched on the second day. As mentioned above, survival of larvae in all treatments in experiment 2a was greater than 80%, the minimum acceptable control survival for a valid experiment. In contrast, survival in two of the three food treatments in experiment 2b, in addition to the concurrently run ASTM no-food treatment, was below 50%. During both experiments 2a and 2b, it was noted that the overall condition of the larvae from the second day's hatch (ie those used in experiment 2b) appeared to be worse than the first day's hatch, in terms of general activity and motility (S Williams pers obs). As a result, the day of hatch was suggested as a possible reason for the inconsistency in larval survival between experiments. It was therefore recommended that for future tests, only the first batch of larvae to hatch from an overnight spawning period be used. This was the approach adopted in experiment 3, and resulted in 90% of the unfed control larvae surviving over 96 h, apparently confirming the suggestion.

In experiment 2b, Aquasonic® fry food had a marked effect on survival of rainbowfish larvae that were collected from the second day's hatch, and were generally in poor condition to start with. This result indicated that the larvae were able to utilise the exogenous food source in order to increase energy supplies and hence improve their overall condition. It is difficult to conclude the reasons why the other two food types were ineffective, other than differences in their availability and/or suitability to the larvae (eg particle size, nutritional status). Inspection of the ingredients and crude analyses of Wardley's® and Aquasonic® fry foods revealed few distinct differences that could account for the differences in survival.

As small doses (15 µl per 30ml) of Aquasonic® fry food were found to significantly increase the chances of larval survival over 96hrs, the copper toxicity experiment (experiment 3) was designed incorporating a feeding component. Feeding had a striking effect on the toxicity of copper to larval black-striped rainbowfish Larvae fed on Aquasonic® fry food showed no response to any of the copper concentrations (0, 1, 3.2, 10, 32, 100 µg/L). The survival of unfed larvae appeared to decrease with increasing copper concentration, with the effect being statistically significant at 100 µg/L. The presence or absence of food has been shown to influence the effect of various toxicants on a range of organisms including some fish species (Holdway & Dixon 1985; Holdway et al 1987; Holdway & Dixon 1988) and invertebrates (Chandini 1988; Kungolos & Aoyama 1993; Klüttgen & Ratte 1994; Barry et al 1995). The results of the present study showed that in the presence of food, copper as a toxicant had no effect on the survival of larval black-banded rainbowfish at the concentrations tested. Without food, larval fish survival decreased with increasing copper concentrations.

Metabolic costs of detoxication have previously been recognised as a possible reason for enhanced organism survival in the presence of a food source (Holdway & Dixon 1985; 1987). In the absence of a sufficient food source to offset the metabolic costs of detoxification, death then results from starvation (Holdway & Dixon 1985; 1987). Nutritional status has also been shown to affect the responses of some species of *Daphnia* to heavy metals (Kungolos &

Aoyama 1993; Klüttgen & Ratte 1994). In contrast to studies using fish, the presence of food has been shown to have a detrimental effect on cladocerans in the presence of particular toxicants (Kungolos & Aoyama 1993; Klüttgen & Ratte 1994; Barry et al 1995). Barry et al (1995) found a negative correlation between the toxicity of the pesticide, endosulfan, and concentration of an algal food source for *Daphnia carinata*. The pesticide was found to adsorb to the food source, increasing the exposure, and hence, toxicity, to *D. carinata*. This evidently was not the case with Cu and larval rainbowfish in the present study, as feeding enhanced survival of the larval rainbowfish over 96 h.

Rainbowfish are considered to be relatively tolerant to copper exposure in comparison to other fish and invertebrate species (Baker & Walden 1984; Williams 1984), although in general, fish and invertebrates are often more sensitive to copper compared many other heavy metals (Baker & Walden 1984). Williams (1984) also suggested that much of the copper in solution would be adsorbed or complexed, and therefore unavailable to the fish as a toxicant.

Many abiotic and biotic factors have been suggested, and found, to interfere with toxicants, depending on the toxicant and what else is present in the experimental medium. As well as interactions between the toxicant and other compounds or materials, the concentration of toxicant and duration of exposure are important contributing factors (Kungolos & Aoyama, 1993). The size, age and health of fish have also been found to play an important role in toxicity effects (Baker & Walden, 1984; Holdway & Dixon, 1985). Smaller or more juvenile fish have been found to be more sensitive to toxicants. The present study assessed newly hatched larval rainbowfish, as they would presumably be more sensitive to toxicants.

From the results of experiment 3, the no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) of copper for the black-striped rainbowfish (*M. nigrans*) were concluded to be 32 and 100 µg/L, respectively, based on larval survival over 96 h. However, larval survival at 32 µg/L was very close to being statistically significant compared to the control treatment (P = 0.06), indicating that the LOEC may actually tend towards 32 µg/L rather than 100 µg/L. In fact, the power of the experiment, in terms of effects of copper exposure was calculated to be approximately 0.65, resulting in a 35% chance of committing a Type II error (β), or failing to detect a difference when in fact one existed (see Zar 1984). This most likely explained why the 23% reduction in survival at 32 µg/L copper (unfed) was not found to be statistically significant. Further tests, using a greater number of replicates, and focussing on copper concentrations between 10 and 100 µg/L (eg 0, 10, 15, 20, 30, 40, 60 and 80 µg/L), could be carried out in order to refine the above estimates of the LOEC and NOEC.

Throughout the series of tests conducted within this study, there was a general increase in survival of control treatment larvae, particularly noticeable from the preliminary tests (results unpublished). The brood stock of adult rainbowfish were initially taken from a larger brood pond where they had been maintained on a diet of Wardley's® flake food and other naturally occurring food sources in the natural water in which they were maintained. When isolated in breeding aquaria for spawning they were fed initially with Wardley's® flake food three times daily. However, they spawned poorly when first established in the separate breeding aquaria. Subsequently, their diet was changed to Barastoc® No. 2 crushed fish pellets twice a day and decapsulated brine shrimp (*Artemia* sp.) once a day (for details refer to Appendix A). Egg

production dramatically increased and larval survival was enhanced following this diet change. Thus it was apparent that the health and diet of the adult fish was directly related to the 'quality' and fitness of their offspring, and the variability observed.

It was initially anticipated that the relative sensitivity of *M. nigrans* larvae to copper be compared to that of purple-spotted gudgeon (*M. mogurnda*) larvae, in order to further assess their suitability as a toxicity testing organism. However, this could not be achieved within the time-frame of the present study. Markich & Camilleri (1997) reported the EC_{50} (survival) and BEC_{10} (analagous to NOEC) of *M. mogurnda* larvae to copper to be 22.7 and 12.2 µg/L, respectively. These values are substantially lower than would be estimated for *M. nigrans* based on the data from the present study. However, the experiments of Markich & Camilleri (1997) were carried out in an extremely soft (hardness ~ 1 mg $CaCO_3$/L; conductivity < 20 µS/cm) synthetic water with low complexing and buffering capacity, in which most metals would be expected to exhibit greater toxicity. In contrast, the toxicity experiment in the present study was carried out using soft ASTM reconstituted water, with a hardness of 40-50 mg $CaCO_3$/L, and conductivity of approximately 370 µS/cm. Thus the apparent differences in toxicity of Cu to both fish species may be largely due to the different test media used.

Although *M. nigrans* offers several laboratory culturing and testing advantages over *M. mogurnda*, as outlined in section 1, it should not be adopted as a replacement fish species to *M. mogurnda* until a proper assessment of their relative senstivities to toxicants relevant to the wet-dry tropics of northern Australia (eg heavy metals, pesticides, complex effluents from mining operations) is undertaken. However, where *M. nigrans* is used for *in situ* toxicity testing purposes at ERA's Ranger uranium mine, a laboratory test using larval *M. nigrans* would be extremely beneficial, providing continuity between the field and laboratory assessments.

5. Conclusions

The major purpose of the present study was to assess whether larval black-striped rainbowfish (*M. nigrans*) would be a suitable test species (and life stage) for use in standard laboratory acute toxicity tests. Given appropriate husbandary/culturing techniques for adult brood stocks, large numbers *M. nigrans* larvae were produced that demonstrated 80 - 100% survival over a standard 96 h (4 day) period under laboratory testing conditions. Larvae were also able to survive the 96 h period without being fed, an important requirement in acute toxicity testing. However, again, this was dependent on adequate husbandary of the adult brood stocks. Acute toxicity of black-striped rainbowfish to copper was demonstrated, with a NOEC and LOEC of 32 and 100 µg/L (nominal), respectively, based on survival following a 96 h exposure period. However, feeding greatly decreased coppper toxicity to rainbowfish larvae, with no effect on survival being observed up to the highest concentration of 100 µg/L.

The present study demonstrated that *M. nigrans* is a suitable fish species for standard acute toxicity testing under laboratory conditions. While the relative sensitivity of *M. nigrans* and *M. mogurnda* to relevant toxicants should be assessed prior to the adoption of the former as a standard toxicity testing organism, its use for laboratory toxicity testing in conjunction with its current use for *in situ* testing of mining wastewaters at Ranger is recommended.

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Appendix A

Husbandry method for *Melanotaenia nigrans*

Brood Stock

Black-striped rainbowfish (*Melanotaenia nigrans*, Richardson) are distributed along the northern part of the Northern Territory and Cape York Peninsula (Allen & Cross 1982). The local distribution is throughout the upper ranges of the lowland region and escarpment area of the Magela Creek system. The experimental animals are bred from laboratory stocks initially collected from Magela Creek and Burdulba Creek, over a number of years. Wild fish are added to the laboratory breeding stock each year to maintain genetic diversity and overall health of the population.

Eight 50-60 litre glass aquaria are required, lined with washed gravel and under-gravel filters. The tanks were filled with tap water and left with the filters overnight to prepare the water for the addition of fish. Four females and two males were placed in each tank, from the main breeding stock population. *M. nigrans* are sexually dimorphic, the females growing to about 60 mm in length and the males 70 mm (Merrick & Schmida 1984). Males have a stronger dark stripe than do females. The pelvic fin of males is more pointed at the posterior end and has a dark edge.

The aquaria can be heated to 28°C for 2-3 hours in the mornings, in cooler weather (Dry season). This unecessary during the 'build-up' and wet season. Fish should be fed three times daily with a variety of food types: Barastoc® No. 2 crushed fish pellets in the morning and afternoon, and live brine shrimp nauplii (*Artemia* sp.) at midday. This diet can be supplemented with flake food, dried blood worms and a selection of live food such as chironomid and mosquito larvae. It is well known that a varied diet is important for the health of fish (Merrick & Schmida 1984). To maintain water quality, aquarium water should be changed weekly, 30% water change at one time using a wide-mouth vacuum siphon. The siphon acts as a vacuum and gravel should be gently disturbed allowing excess food and waste products to be removed. The glass sides within the aquaria should be wiped down to hinder algal and flatworm blooms. Regular maintenance of aquaria and regular feeding with varied food types are essential for healthy fish and thus good productivity.

Production of larvae

Water weeds or plastic plants need not be kept in the rainbowfish aquaria as it is believed this induces them to spawn. Woollen mop spawning substrates are placed in the aquaria and the fish spawn onto these. They can then be removed and placed into separate incubation tanks, as adult rainbowfish will eat their newly hatched larvae. Rainbowfish spawn daily over the Wet season (Boyden et al 1995) and continuously all year round. Development time of larvae from egg-laying to hatching is 4-6 days at 30°C. Spawning mops should thus be placed in aquaria (two in each) the appropriate number of days before larvae are required for testing.

Suitable incubation tanks are 90L Nally bins, cleaned thoroughly and 3/4 filled with tap water. A water heater calibrated to 30°C and an aerator with an activated carbon filter are

placed in the incubation tank, and with the lid in place the tank is left to equilibrate over night. When the spawning mops have been in the adult brood aquaria overnight, they are removed in the morning and placed into the incubation tanks.

Collection and selection of larvae for testing

Larvae from an overnight spawning period generally hatch over two days (or longer). It is best to use first day larvae close to <12 hours old. If there is a problem obtaining enough larvae to begin an entire test from one night's spawning, then pool eggs from two overnight spawning periods and use the collective larvae hatched on the morning you require larvae (it is important that the larvae are similar hatching age so it is necessary to remove any larvae hatched the day before, from the incubation tanks). When the test has commenced, the remaining larvae are placed into a rearing aquarium, or disposed of using the standard anaesthetic MS222. The incubation tanks should be emptied, thoroughly washed out, then refilled for the next batch of eggs. The spawning mops are washed with *Nufarm 'Iodox' iodine disinfectant and rinsed completely, then hung to dry. This cleaning procedure is important in maintaining a high standard of cleanliness and reducing chances of fungus and disease transmission.

Appendix B

Synthetic water recipes

eriss synthetic water

The synthetic water is made up with Milli-Q water in a 5000 ml volumetric flask the day before use, made up to 20L into a plastic barrel and aerated with an air-stone bubbler over night. It is buffered to a pH of 6.0 ± 0.15 with 0.05 M H_2SO_4 or 0.05 M NaOH.

Reagent	Stock solution concentration (g/L)	Volume added to 20 L MilliQ water (ml)
$NaHCO_3$	82.2	0.88
$Al_2(SO_4)_3.18H_2O$	19.615	0.88
$MgSO_4.7H_2O$	138.1	0.88
$CaCl_2.2H_2O$	37.46	0.88
KCl	16.01	0.88
$FeCl_3.6H_2O$	68.09	0.147
Trace Element solution		0.44

ASTM reconstituted fresh water

The reconstituted water is made up with Millli-Q water, in a 5000 ml volumetric flask the day before use and is buffered to a pH of 6.5 ± 0.15 with 1.0 M NaOH solution and 1.0 M KH_2PO_4 solution.

Reagent	Concentration required for soft water (mg/L)
$NaHCO_3$	48
$CaSO_4.2H_2O$	30
$MgSO_4$	30
KCl	2

To adjust pH to 6.5 ± 0.15, add the following to 15L of the above soft water:

Reagent	Volume required for pH 6.5 ± 0.15 (ml)
1.0 M NaOH	5
1.0 M KH_2PO_4	30

For further details regarding ASTM reconstituted fresh water, refer to ASTM (1992).

Appendix C

Ingredients and Guaranteed analyses of commercial fish foods

Wardley's Liquid Small Fry® Baby Fish Food (egglayer formula).

Ingredients: distilled water, egg powder, autolysed yeast extract, vitamin A palmitate, citric acid, sodium benzoate (as a preservative).

Guaranteed analysis: Min. crude protein - 6%; mi. crude fat - 3.5%; max. crude fibre - 0.4%, max. ash - 1%; max. moisture - 88%.

Aquasonic® "Freshwater" Fry Starter

Ingredients: shrimp, fish, egg, sodium alginate, sorbic acid, sodium benzoate, vitamin A.

Guaranteed analysis: unavailable.

Appendix D

Raw data: Number of larvae surviving after 96 h

Experiment 1: Survival of larval *M. nigrans* in different water types

Water type	*Replicate*		
	1	*2*	*3*
eriss synthetic water	2	7	3
ASTM reconstituted water	6	8	3
Buffalo Billabong (filtered)	6	2	7
Buffalo Billabong (unfiltered)	6	4	2

Experiment 2a: Effect of test water volume and feeding

Treatment	*Replicate*		
	1	*2*	*3*
ASTM water - 30 ml	10	8	9
ASTM water - 150 ml	8	7	9
ASTM water (30 ml) + food	6	10	7

Experiment 2b: Effect of food type and ration

Food type and ration	*Replicate*		
	1	*2*	*3*
Algae - 15 ul	3	5	3
Algae - 30 ul*	4	3	2
Algae - 60 ul*	3	4	2
Wardley's fry food - 15 ul	4	6	9
Wardley's fry food - 30 ul	1	0	6
Wardley's fry food - 60 ul	2	5	4
Aquasonic fry food - 15 ul	9	9	10
Aquasonic fry food - 30 ul**	6	6	7
Aquasonic fry food - 60 ul**	6	7	7
ASTM water control (no food)	2	5	4

* started with only 6 larvae

** started with only 7 larvae

Experiment 3: Acute toxicity of copper and the effect of feeding on copper toxicity to larval *M. nigrans*

Cu concentration (ug/L) and fed/unfed	*Replicate*		
	1	*2*	*3*
0 - unfed	10	7	10
1 - unfed	10	7	8
3.2 - unfed	9	9	10
10 - unfed	9	8	9
32 - unfed	7	7	9
100 - unfed	4	3	6
0 - fed*	10	10	10
1 - fed	9	10	10
3.2 - fed	10	10	10
10 - fed	10	9	10
32 - fed	10	10	10
100 - fed	10	10	10

* each replicate was provided with 15 µl Aquasonic® fry food