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The development of a rapid response toxicity bioassay based on the feeding rate of the tropical cladoceran Moinadaphnia macleayi

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supervising scientist

The development of a rapid response toxicity bioassay based on the feeding rate of the tropical cladoceran *Moinodaphnia macleayi*

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Declaration

The work described in this report was performed while I was an enrolled student for the Bachelors of Applied Science (Honours) (Applied Biology and Biotechnology) in the Department of Applied Biology and Biotechnology at the Royal Melbourne Institute of Technology.

To the best of my knowledge, all the work performed by others, published or unpublished, has been duly acknowledged in the report.

This report has not been submitted, in whole or in part, for any reward.

Signed: .....

Date: .....

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Abstract

Cladocerans are used widely for ecotoxicological bioassays. The most commonly used endpoints are reproduction and survival. Reproduction is a sensitive indicator of toxicity, however reproduction bioassays are lengthy and time consuming tests to perform. Recent research has reported that feeding rate (measured over 24 h) is a sensitive indicator of toxicity for *Daphnia magna*. Using a similar methodology to these tests, a feeding bioassay has been developed for the Australian tropical cladoceran *Moinodaphnia macleayi*, and its sensitivity tested in comparison with the standard reproduction bioassay. Feeding rate has been found to be a more sensitive indicator of toxicity than reproduction for cadmium. No feeding inhibition was detected when test animals were exposed to copper, despite significant test animal mortality. The difference in feeding and reproductive responses following cadmium and copper exposures suggest different dominant modes of toxicant action. No feeding inhibition was detected for uranium at concentrations ≤290 µg/L. The feeding bioassay was validated on 2 separate samples of multi-toxicant mine release water. Results demonstrated that a feeding biossay performed in 20 h has comparable sensitivity to a reproduction bioassay performed over 5-6 d.

Contents

List of Abbreviations

ANOVA - analysis of variance

ARR - Alligator River Region

ASTM - American Society for Testing and Materials

CI - confidence interval

CV - coefficient of variation

DOM - dissolved organic matter

EC5 / 20 - concentration causing a 5 / 20 % effect in test organisms relative to the control

ERA - Energy Resources of Australia

eriss - Environmental Research Institute of the Supervising Scientist

FFV - fermented cichlid food

LC50 - concentration lethal to 50 % of the test organisms relative to the control in a given time

LOEC - lowest observed effect concentration

NOEC - no observed effect concentration

NT - Northern Territory

OECD - Organisation for Economic Co-operation and Development

POM - particulate organic matter

SE - standard error

SEA - seaweed extract additive

USEPA - United States Environmental Protection Agency

α - alpha (level of significance)

°C - degrees Celsius

cells/ml - cells per millilitre

d - day

h - hour

km - kilometre

l - litre

µg/l - micro-gram per litre

µl - micro-litre

µm - micro-metre

µS/cm - micro-siemen per centimetre

min - minute

mg/l - milligram per litre

mm - millimetre

r - intrinsic rate of natural increase of a population

$\mathbf{t_{0/20/24}}$ - at time = 0 / 20 / 24 hours

y - year

Cd - cadmium

Cu - copper

U - uranium

< - less than

≤ - less than or equal to

> - more than

≥ - more than or equal to

% - percentage

± - plus or minus

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Conference Abstracts

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Chapter One: Introduction

1.1 The Use of Ecotoxicological Bioassays

1.1.1 The need for assessing environmental impacts - an historical perspective

Over the last 30 to 40 y, a marked increase in the number of chemicals released into the environment has occurred, causing great concern about their possible effects on natural systems. Subsequent adverse effects in aquatic systems receiving waste water prompted environmental management both to document apparent damage, and also to quantify environmental risk. From these initiatives grew the role of standardised test protocols in ecotoxicology, to enable the detection, monitoring and control of chemical pollution by regulatory authorities and industry.

Toxicity tests are one approach that measures the response of living organisms to various contaminants. They are used for the: a) prediction of environmental effects of waste, b) comparison of toxicants or animals or test conditions, and c) regulation of discharge (Buikema Jr. *et al.* 1982). Conducting toxicity tests in the natural environment is generally unacceptable for ethical and logistical reasons, hence the development of controlled laboratory toxicity tests, usually on single species. Estimates of environmental damage are then extrapolated from the organism level of biological organisation to whole natural systems, requiring composite modelling (Kooijman *et al.* 1989; Gurney *et al.* 1996). Understanding the complexity of these relationships represents one of the key research challenges of ecotoxicology (McCauley *et al.* 1990; Calow *et al.* 1997; Parker *et al.* in press).

1.1.2 Application of laboratory bioassays to the field

Population level effects in some species can be predicted from information from toxicity tests by the use of simplified life-history models (Sibly 1996; ASTM 1997; Calow *et al.* 1997). This requires age-specific survival and fecundity rates to be derived from life table experiments, from which r (intrinsic rate of natural increase of a population) can then be calculated (Van Leeuwin *et al.* 1985; Calow *et al.* 1997). Therefore, it is important to establish to what extent specific effects on life-history variables influence

population growth rates, and the level of sensitivity of population growth rate to changes in each of the life-history characteristics (Calow *et al.* 1997). At the level of the individual, it is also necessary to investigate what processes within the organism are responsible for changes in life-history traits, and how these processes will be affected by toxic stress (Kooijman 1986; Calow and Sibly 1990; McCauley *et al.* 1990; Bradley *et al.* 1991; Sibly 1996).

1.1.3 Toxicity bioassays – applications and problems

In recognition of the magnitude of time and expense required to perform life-history experiments for a broad suite of organisms, rapid acute and more lengthy chronic tests have been developed for a range of 'indicator' species. These comprise the only 2 test designs commonly used in ecotoxicology (Buikema Jr. *et al.* 1982). The acute test usually only measures mortality as an endpoint, while the chronic test measures parameters such as mortality, growth and reproductive success. The value in the acute test lies in its rapid completion, simplicity and unambigous endpoint (i.e. death). Results can provide meaningful comparisons of toxicant lethality between organisms, toxicants or test conditions. However, acute tests do not measure sub-lethal effects, and therefore cannot readily be used to predict sub-lethal toxicant concentrations able to harm a population or ecosystem (Buikema Jr. *et al.* 1982; Roux *et al.* 1993). Chronic tests, that measure sub-lethal responses to toxicant exposure, are more ecologically relevant as they predict concentrations which may affect a population's growth rate (Van Leeuwin *et al.* 1985). However, these tests are more time-consuming and expensive to perform. In addition, chronic tests have been criticised for a lack of physical, chemical and biological realism (Buikema Jr. *et al.* 1982), as well as neglecting effects from interspecies interactions, feedback mechanisms, biomagnification or recovery from stress (Parker *et al.* in press). Nonetheless, results of single species chronic tests have correlated well with larger mesocosm tests in terms of predicting toxic chemical concentrations. However, they appear limited in their ability to estimate the magnitude of environmental harm, particularly due to indirect effects (Borgmann *et al.* 1989; Jak *et al.* 1996; Van den Brink *et al.* 1996). Complexity and expense are the major problems associated with mesocosm testing (Parker *et al.* in press).

Current toxicity bioassays also fail to fully address responses such as changes to organism energy allocation. These effects may manifest in the form of energy trade-offs and alterations in reproductive strategy. For example, an organism exposed to a stressor may reach reproductive maturity at an earlier or later age than under 'normal' conditions, translating to significant population level effects. The importance of such a response may be underestimated in the interpretation of reproduction bioassay results (Van Leeuwin *et al.* 1985).

Hormesis (a stimulatory effect from exposure to trace levels of metals) (Stebbing 1981) is another response reported as a result of toxicant exposure (Winner and Farrell 1976; Bodar *et al.* 1988b; Roux *et al.* 1993). An increase in offspring number is generally interpreted in reproduction bioassays as a positive result. However, it is possible that such an effect is the result of a trade-off, rendering the adult, or its offspring, in a more vulnerable physiological state (Bodar *et al.* 1988b).

1.1.4 Possible improvements to toxicity testing regimes

The following recommendations have been put forth to improve our ability to formulate relationships between individual organism responses and possible ecosystem effects:

- Simple and rapid tests should be developed to enable testing of a large array of species (Baudo 1987; Calow and Sibly 1990; Janssen and Persoone 1993; Juchelka and Snell 1995; Bitton *et al.* 1996).
- Ecotoxicological bioassays should be developed locally (Buikema Jr. *et al.* 1982; Roux *et al.* 1993; Markich and Camilleri 1997).
- Ecophysiological characteristics of species should be investigated in order to recognise organismal processes which may signal effects at a higher level (Buikema Jr. *et al.* 1982; Van Leeuwin *et al.* 1985; Calow and Sibly 1990; Koeman 1991; Sibly 1996; Van den Brink *et al.* 1996).
- Factors (both abiotic and biotic) which will potentially modify responses measured in the laboratory should be investigated (Baird *et al.* 1991b; Holdway 1992a, b; Barata *et al.* 1998; Parker *et al.* in press).

- Laboratory toxicity testing should be validated with mesocosm or *in situ* assessment (Baudo 1987; Holdway 1992a, b; Barbour *et al.* 1996; Gurney *et al.* 1996).

1.2 Cladocerans as Test Animals

1.2.1 Appropriateness as test species

Cladocerans are one of the most commonly used organisms in aquatic toxicology (Baudo 1987; Roux *et al.* 1993). This is largely due to fulfilment of criteria considered important by the United States Environmental Protection Agency (USEPA) (1979) in the selection of test species:

1. ecological representativeness (in terms of taxonomy, trophic level, or niche);
2. membership in trophic chains either directly or indirectly (i.e. economically) related to man;
3. availability and suitability for laboratory testing;
4. existence of adequate background data on biology and ecology (Baudo 1987).

Some authors have questioned the relative importance of each of the criteria, and the choice of certain cladoceran species as the most appropriate test animal (Winner *et al.* 1990; Jak *et al.* 1996). However, it is impractical to test a large proportion of species endemic to each system of interest. In addition, organismal responses to toxicant exposure are often chemical-specific preventing clear ranking of species for toxicant sensitivity (Baird *et al.* 1991b; Holdway 1992a, b). Cladocerans are recognised as having general sensitivity to toxicants (Buikema Jr. 1980), short life span (hence high statistical power) (Parker *et al.* in press), importance in the food web (Jones *et al.* 1991), widespread distribution and ease of laboratory handling (Baudo 1987). These characteristics have predicated cladocerans' wide use as toxicity test species.

1.3 The Cladoceran Feeding Model

1.3.1 The need for developing a feeding model

In order to understand the biochemical pathway of toxicants though the environment, it is important to investigate the role that feeding has upon toxicant uptake, and subsequent changes to an organism's metabolic capacity. Feeding is the major form of energy input for all animals, enabling energy allocation towards maintenance, growth and reproduction. Hence, if feeding behaviour is altered by toxic exposure, these functions could be affected (Calow and Sibly 1990; Allen *et al.* 1995; Taylor *et al.* 1998).

Feeding also provides the major energy and nutrient link between trophic levels, hence disruptions to feeding regimes could have cascading effects through the food chain (Jak *et al.* 1996; Van den Brink *et al.* 1996). Foraging patterns will also affect species' survival probabilities though exposure to predators and competition for resources (Calow and Sibly 1990).

1.3.2 Effect of feeding on toxicity in laboratory tests and the field

Substantial research has pointed towards contaminated food sources as being a major route for metal uptake in aquatic organisms (Campbell 1995; Taylor *et al.* 1998). In addition, many studies have found that availability of food, food type, and nutritional status can have significant effects upon an organism's sensitivity to toxicants (Lanno *et al.* 1989; Chandini 1989; Campbell 1995; Taylor *et al.* 1998). Therefore, the significance of food type and consumption in transporting or ameliorating the uptake of metals is important to understand because it could modify the interpretation of laboratory results and the prediction of environmental impacts.

1.3.3 Toxicant modes of action

Calow and Sibly (1990) suggested that the commonly used index of environmental stress, 'scope for growth', will have differing consequences on r, depending on whether any reduction has been brought about by a reduced in food intake, or an increased metabolic cost of synthesis. Barber *et al.* (1990) referred to these modes as 'supply-sided' effects and 'demand-sided' effects, respectively. They later found that in a 24 h period, oxygen consumption decreased in cadmium-exposed clones of *Daphnia magna*, a symptom

not expected if increased demands were being made on the body as a result of synthesis of proteins due to chemical damage (Barber *et al.* 1994). It appeared that the primary effect of cadmium was to reduce feeding, resulting in a reduction in weight gain. They did not, however, discount the importance of demand-sided effects over longer time periods, as shown by other research (Baird *et al.* 1990; Barber *et al.* 1990).

1.3.4 Investigating the mechanics of toxicity

Allen *et al.* (1995) and Taylor *et al.* (1998) measured feeding inhibition in *D. magna* as a result of cadmium toxicity, and put forward 3 possible hypotheses for the mechanism of toxicity:

1. Direct effect upon the animals feeding apparatus (e.g. filtering mechanism, thoracic appendages) from contact with contaminated food particles.
2. Animal may reject the food once it has been successfully collected i.e. the animal may be able to 'taste' the toxicant. Filtering rate may remain unaffected.
3. Contaminated cells are collected and ingested normally, but absorption is reduced due to the toxicant unbinding in the gut, subsequently interfering with digestion, and allowing cells to pass though the gut intact and undigested.

Allen *et al.* (1995) proposed that (3.) was the likely mechanism while Taylor *et al.* (1998) suggested either (1.) or (3.). Support for all 3 theories can be found in the literature. Griffiths (1980) found that after 2 h of exposure to cadmium, the gut diverticulum of *D. magna* were paralysed, shrunken and empty of chlorophyll, supporting 'gut poisoning' as the dominant mode. Bodar *et al.* (1988a) measured consumption and assimilation rates in *D. magna* and found that while consumption decreased following exposure to cadmium, assimilation rates only decreased by an amount explained by the reduction in food intake. Digestion and absorption were only marginally affected, and while no single metabolic process appeared particularly depressed, metabolic activities seemed generally inhibited. Chandini (1989) observed that 'green gut' (visible presence of food in the gut) was absent when *D. carinata* was exposed to cadmium, suggesting that ingestion (but not necessarily assimilation) had been inhibited.

Research supporting the hypothesis of 'damaged feeding mechanisms' has involved tethering, filming and visually observing *Daphnia* exposed to crude oil (Wong *et al.* 1983) and sodium dodecyl sulphate (Jones *et al.* 1991). Both papers reported significant reductions in filtering rates during exposure. Wong *et al.* (1983) also reported that the rejection rate of ingested food increased during exposure. There are possibly several mechanisms of toxicity for any organism, and the dominant mode may vary between organism and toxicant.

1.3.5 Energy allocation models

Once the dominant mode of toxicant action has been established it is then possible to estimate potential population effects with the use of individual energetics models (Schindler 1968; Gurney *et al.* 1996; Sibly 1996). Four alternative models of energy allocation for *D. magna* are:

- Kooijman (1986): Assimilate initially enters a reserve compartment and energy is partitioned between growth, maintenance, and reproduction. Maintenance is at the expense of growth when the animals are growing, and of reproduction, when they are not. Allocation to growth or reproduction will be reduced only enough to satisfy requirements for maintenance. Hence death will follow shortly after cessation of reproduction.
- McCauley *et al.* (1990): Energy is allocated directly for maintenance, growth or reproduction. Under food limitation, reproduction and growth will cease immediately, but stored energy will sustain maintenance, hence death will not be imminent.
- Bradley *et al.* (1991): Assimilated energy enters stores in addition to direct allocation to growth and reproduction. Under food limitation, allocation to reproduction will cease; however, maintenance and then growth will be supported from stored energy. Death will not be imminent upon starvation, although a reduction in reproduction will be apparent within one instar.
- Glazier and Calow (1992): Energy allocation rules will vary between organism clone and age, as well as food availability. Maintenance is always the highest priority.

Following the model of Bradley *et al.* (1991), if the dominant toxicant mode of action is supply-sided, then fecundity will decline within one instar of exposure due to a reduction in food intake. Hence, feeding inhibition or reproductive parameters measured over one instar will provide a sensitive testing endpoint. If however, the effects are demand-sided, then increased maintenance costs will reduce the amount of stored reserves available for growth, hence growth rather than reproductive traits or feeding should be measured. For this reason, a battery of short tests has been recommended as the most effective method of toxicity testing (C. Barata pers. comm.).

1.4 Development of a Cladoceran Feeding Test

1.4.1 Rationale

Many authors have suggested feeding rate as a sensitive endpoint for cladoceran toxicity tests (Cooley 1977; Reading and Buikema Jr. 1980; Flickenger *et al.* 1982; Wong *et al.*1983; Bodar *et al.* 1988a; Jones *et al.* 1991; Allen *et al.* 1995; Juchelka and Snell 1995; Bitton *et al.* 1996; Jung and Bitton 1997; Taylor *et al.*1998; C. Barata pers. comm.). Rationale for using feeding inhibition as an endpoint includes rapid assessment, and physiological and ecological relevance.

1.4.2 Methods of testing

Using several methods, feeding rates have been measured with varying success and sensitivity. Wong *et al.* (1983) and Jones *et al.* (1991) tethered and filmed toxicant-exposed cladocerans. While this observational approach may be helpful in explaining some of the mechanistic effects of toxicant-stressed animals (e.g. afflicted appendages and rejection rate of food), there are problems with using these methods for a standard bioassay. Primarily, Jones *et al.* (1991) remarked that tethering significantly affected organism movements and filtering rates. In addition, this method only measures filtering rate, rather than consumption rate.

Feeding rate has also been measured by feeding fluorescent stained material (e.g. beads or yeast) to pre-exposed animals (Flickenger *et al.* 1982; Bodar *et al.* 1988a; Juchelka and Snell 1995; Bitton *et al.* 1996; Jung and Bitton 1997). The endpoint was presence of fluorescence in the gut. Janssen and Persoone (1993) pointed out that intermediate levels of fluorescence in the gut had to be recorded as 'present' or 'absent',

hence the scoring system was somewhat subjective. However, results of the above test correlated well with sensitivities from acute tests.

A challenge inherent in developing laboratory bioassays is to imitate natural conditions. Despite reasonable correlations of the abovementioned feeding tests with currently used bioassays, unrealistic feeding regimes, and the large degree of manipulation of the test organisms, both during and after feeding, makes confounding of results a significant problem. In addition, the responses measured carry minimal ecological relevance, hence only serve use as comparisons within the boundaries of the test.

Feeding inhibition has also been measured by calculating changes in ambient algal cell concentrations of toxicant-exposed animals (Allen *et al.* 1995; Taylor *et al.* 1998; C. Barata pers. comm.). This method involves use of a more authentic food source (hence incorporating more realistic levels of metal adsorption, feeding rate, and desorption in the gut), objective measurement of a continuous function, and very little manipulation of test animals or food. Therefore, the measured feeding rate is less likely to be confused with any other possible factor. This method has been successful in terms of ease or performance, and test sensitivity (Allen *et al.* 1995; Taylor *et al.* 1998; C. Barata pers. comm.).

1.5 Application to Australian Tropical Species - Moinodaphnia macleayi (Crustacea, Cladocera)

Microcrustaceans are an important component of the aquatic ecosystem in the Alligator Rivers Region (ARR) of Australia's Northern Territory (NT) (Julli 1986). They are able to take advantage of the high detritus, bacteria and phytoplankton load as a food source, and in turn they form a sizeable portion of the diet of the numerous fish species (Bishop *et al.* 1980). Cladocera appear particularly abundant in this region; 43 species having been described from the Magela Creek flood plain alone (Julli 1986). *Moinodaphnia macleayi* (Smirnov and Timms 1983) (Figure 1) are from a monotypic genus and occur in weed-bed habitat (Figure 2) in coastal NT, Queensland and New South Wales (Julli 1986).

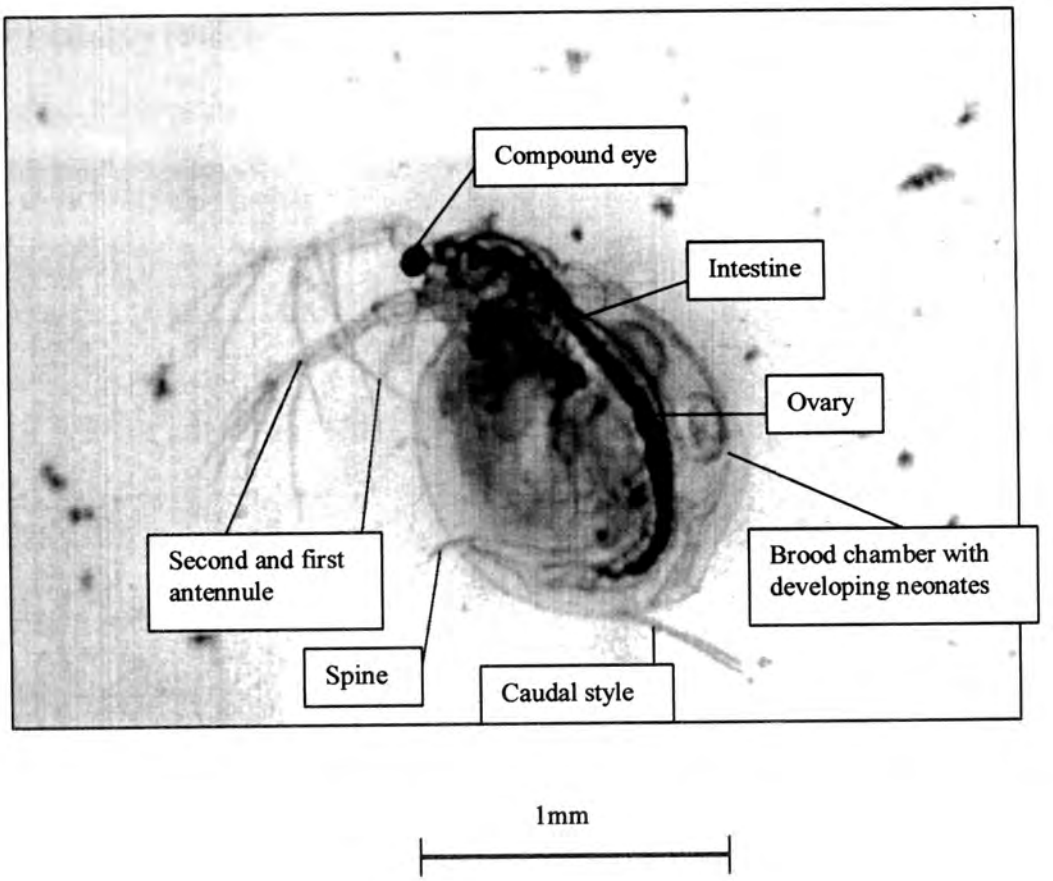

Figure 1. Photomicrograph of *Moinodaphnia macleayi.*

Figure 2. Photograph of typical *M. macleayi* habitat - Bowerbird Billabong (Western Arnhem Land).

Holdway (1992a) assessed 19 freshwater species from the ARR, and on the basis of breeding and rearing success, selected *M. macleayi* as 1 of the 8 species with excellent potential as a test organism. The species' short life-cycle (5-7 d) was considered a positive feature, although it was also acknowledged that the organism was very sensitive to culture conditions (Holdway 1992a). Julli *et al.* (1990) also encountered culturing difficulties with *M. macleayi*, citing unacceptably high mortality (OECD 1987) as its major drawback as a test organism. However, an improved diet of *Chlorella* sp. (at 2×10^5 cells/ml) with fermented cichlid food (FFV) (Appendix 18) has been found to increase fecundity and survival (Hyne *et al.* 1993).

Since then, 2 toxicity bioassays using *M. macleayi* (with endpoints adult mortality and reproduction) have been developed as recommended protocols for regulation of water release from Ranger Uranium Mine (Hyne *et al.* 1996). The successful use of *M. macleayi* for pre-release waste water toxicity testing in the ARR makes it a suitable cladoceran for further development of toxicity bioassays. In addition, the ARR contains wilderness areas recognised on the World Heritage List, as well as being a key operational area for Australian mining industries. These factors reiterate the need for ongoing ecotoxicological research in the area; both refining procedures and protocols to increase their sensitivity and application, and also to gain a greater understanding of local ecological processes.

1.6 Metals of Regional Interest

The ARR lies within one of Australia's richest mineral deposits. Since 1865, 16 metals have been extracted from the area. Today, the ARR is heavily mined for uranium, however deposits of other metals have also been identified. The large size of the East Alligator River uranium field suggests that it will remain the metal of most interest for local mining industries. The removal of uranium also entails extraction of many other metals of concern to ecotoxicologists, including aluminium, cadmium, cobalt, copper, manganese, lead and zinc. Largely as a result of mining activities, these metals (and including uranium) have been identified as priority metals of potential ecotoxicological concern in aquatic ecosystems of tropical Australia (Markich and Camilleri 1997).

1.6.1 Selection of metals for investigation

Cadmium exposure is known to induce feeding inhibition in *D. magna* (Allen *et al.* 1995; Taylor *et al.* 1998; C. Barata pers.comm.). Therefore it is an appropriate metal to trial for feeding effects on *M. macleayi*. Copper was also chosen for testing, due to recommendation for further ecotoxicological study in tropical waters of Australia (Markich and Camilleri 1997). It was also important to test an essential metal in order to examine the efficacy of a feeding test on a range of metal types. Uranium was the third metal chosen for study due to being the metal of most interest in the NT. Responses of *D. magna* and other cladoceran species to copper and cadmium are reported in Tables 1 and 2, respectively.

1.6.2 Copper

Copper occurs in the natural environment mainly as sulfides, oxides, carbonates, arsenides and chlorides (Mance *et al.* 1984). Copper is an essential metal, however it can be toxic to some species at concentrations not much higher than those that allow optimum growth (Cairns *et al.* 1978). The free hydrated copper ion (Cu^{2+}) together with the hydroxy species, particularly $CuOH^{+}$ and to a lesser extent $[Cu_2(OH)_2]^{2+}$, are generally considered the most toxic inorganic species to aquatic organisms (Andrew *et al.* 1977). In freshwater, the major factors affecting copper speciation are organic carbon, alkalinity and hardness, and pH. Cu^{2+} typically forms strong complexes with natural dissolved organic matter (DOM) which generally reduces copper bioavailability (Campbell 1995). If dissolved copper concentrations exceed the complexation capacity of the DOM in the water, then a marked increase in the copper bioavailability, and hence toxicity, will occur.

1.6.3 Cadmium

In natural surface waters, cadmium occurs predominantly in the divalent form, comprising several inorganic and organic compounds. Cadmium-DOM complexes usually comprise a small component of dissolved cadmium in freshwater systems (French 1986), however, this will increase with increased pH and DOM concentrations. More important is the free hydrated ion (Cd^{2+}) which is primarily responsible for eliciting a toxic response in aquatic organisms (Campbell 1995).

Table 1. Effects of copper on *D. magna* and other cladoceran species.

Reference	Water type	*D. magna*	Other species
Winner and Farrell 1976	natural (130-160 mg/l $CaCO_3$)	• copper stimulatory to brood size up until 80 µg/l, no effect at 80 µg/l • NOEC-LOEC[1] (reproduction test): 40-60 µg/l • least sensitive of 4 species tested to chronic copper stress • *r* largely determined by the interaction between longevity and brood size • sensitivity as an indicator of effect: longevity > r > brood size	• *D. pulex* • Copper stimulatory to brood size up until 60 µg/l, reduced brood size at 80 µg/l • *r* largely determined by the interaction between longevity and brood size • Longevity the most sensitive indicator of effect
Flickenger *et al.* 1982	natural (93-104 mg/l $CaCO_3$)	• NOEC-LOEC (chronic survival test): 10-20 µg/l • cf. NOEC-LOEC (filtration rate, negative phototaxis behaviour and body length): 0-10 µg/l	
Dave 1984	hard synthetic (ISO)	• LC50[2] (48 h acute bioassay - starved): 6.5 µg/l • LC50 (48 h acute survival test - with food): 18.5 µg/l • LC50 (21 d survival test): 1.4 µg/l • LOEC (growth in juveniles after 7 d / in adult females after 21 d): 6.6 µg/l • reproduction (after 21 d) stimulatory peak at 1 µg/l, but accompanied by reduced survival from 0.4 µg/l	
Baird *et al.* 1991b	hard synthetic (ASTM)	• LC50 (acute survival test) range for 5 clones: 10.5 - 41.2 µg/l	

Reference	Water type	*D. magna*	Other species
Koivisto 1992	NR	• animals smaller with exposure; stronger effect with low food • low food effect greater than Cu effect • effect on carapace size • no effect of Cu on age of maturity but an effect of food quantity • Cu did not affect clutch size or offspring number • high food/ low Cu increased population growth rate	*D. pulex* • Animals smaller with exposure; stronger effect with low food • low food effect greater than Cu effect • no effect of Cu on age of maturity but effect of food quantity • Cu did not affect clutch size or offspring number, but an effect of food quantity • Cu affected growth rates at low food but not at high food *Bosmina longirostris* • Highest sensitivity to Cu than *D. magna* or *D. pulex* • Delayed growth with Cu exposure • No effect of Cu on age of carapace moult, but effect from low food • Delayed reproduction, but effect reduced at high food level • Offspring number affected by food level and Cu and interaction • Population growth rates lower for Cu treatments at both food levels *Chydorus sphaericus* • High Cu and low food caused low growth, but growth not affected for other combinations • Effect of low food or Cu on moulting age for last instars • Offspring number significantly affected by Cu and food level and interaction • Lower population growth rates for Cu treatments at both food levels *D. galeata* • Animals smaller with Cu exposure; stronger effect with low food • low food effect greater than Cu effect • No effect of Cu on age of maturity but effect of food • No effect of Cu on clutch size or offspring number • Population growth rate reduced by Cu at high food level, but increased at low food
Janssen and Persoone 1993	hard synthetic (USEPA)	• $EC50^3$ (1 h observation of enzymatic process): 230 µg/l, cf. LC50 (24 h acute survival test): 280 µg/l, and LC50 (48 h acute survival test): 140 µg/l	

Reference	Water type	*D. magna*	Other species
Roux *et al.* 1993	moderate-hard synthetic (USEPA) (80-90 mg/l $CaCO_3$)		*D. pulex* • LC50 (48h acute survival test): 21 µg/l • NOEC-LOEC (21 d reproduction test): 0.003-0.3 µg/l, NOEC 0.4 µg/l • β type toxicity expected - survival and reproduction higher initially (0.003-3 µg/l) then declined
Juchelka and Snell 1995	hard synthetic (USEPA)		*Ceriodaphnia. dubia* • NOEC (1 h feeding test using fluorescent beads): 30 µg/l cf. NOEC (reproduction bioassay): 20 µg/l
Koivisto 1995 -	NR		*D. pulex* • only effect of Cu was minor delay in maturation *Bosmina longirostris* • same concentrations had impacts on survival, growth, maturation age and fecundity
Bitton *et al.* 1996	moderate-hard synthetic (USEPA)		*C. dubia* • EC50 (1 h CerioFAST feeding test): 14 µg/l, cf. LC50 (48 h acute survival test): 11 µg/l
Barata *et al.* 1998	range of soft to hard synthetic	• LC50 (48 h acute survival test) range for 4 clones in moderate to hard water: 3.2 - 15.4 µg/l; and hard water: 23.1 - 119 µg/l	

* Not reported
[1] NOEC: no-observed-effect-concentration; LOEC: lowest-observed-effect-concentration
[2] LC50: concentration lethal to 50 % of the test organisms relative to the control in a given time
[3] EC50: concentration causing a 50 % effect in test organisms relative to the control

Table 2. Effects of cadmium on *D. magna* and other cladoceran species.

Reference	Water type	*D. magna*	Other species
Van Leeuwin *et al.* 1985	hard synthetic (200 mg/l $CaCO_3$) (Dutch Standard NPR 6503);; and natural (224 mg/l $CaCO_3$)	• NOEC-LOEC in synthetic water (r_m - rate of population increase based on age-specific survival and fecundity): 1.0-1.8 µg/l • r_m for the first 21 d exceeded 99 % of r_m for entire life-table • reduced toxicity in lake water cf. synthetic water • NOEC-LOEC for lake water (r_m): 3.2-10 µg/l; cf. (length): 0-0.32 µg/l • carrying capacity reduced at all concentrations - NOEC for carrying capacity cannot be established	
Bodar *et al.* 1988a	synthetic (150 mg/l $CaCO_3$) (Dutch Standard NPR 6503)	• consumption and assimilation rates significant after 14 d at 5 µg/l • no effect upon assimilation efficiency • body weight affected at ≤1 µg/l • no notable effect upon biochemical composition of animals	
Bodar *et al.* 1988b	synthetic (150 mg/l $CaCO_3$) (Dutch Standard NPR 6503)	• LC50 (25 d reproduction test): 10.0 µg/l • LOEC (delayed reproduction): 5.0 µg/l • increased average number of neonates for 0.5, 1.0 and 5.0 µg/l, however, size of neonates decreased significantly with increase in Cd	
Chandini 1989	pH 7.0		*D. carinata* • high food density increased survival cf. with low and medium food densities • growth significantly affected by Cd but not food density • fertility and fecundity significantly influenced by Cd and food density and interaction
Baird *et al.* 1990	hard (ASTM)	• effect on body length and mass differed only slightly between two clones • when body size effects are factored out, no direct effect upon clutch size or mass for either clone • fitness (capacity for increase) was highly significant between 3 clones at 6 µg/l • relative response to acute and chronic stress was concordant for 3 clones, however convergence in response occurred under chronic stress	

Reference	Water type	*D. magna*	Other species
Barber *et al.* 1990	hard (ASTM)	• increase in respiration rates for 2 clones • no effect upon O_2 consumption or RNA-DNA (indirect measure of protein synthesis) ratios for either clone • rate of protein deposition reduced for both clones	
Baird *et al.* 1991b	hard (ASTM)	• LC50 (acute survival test) range for 5 clones: 3.6 - 115.9 µg/l	
Janssen and Persoone 1993	hard (USEPA)	• EC50 (1 h test -observation of enzymatic process): 410 µg/l, cf. LC50 (24 h acute survival test): 1900 µg/l, and LC50 (48 h acute survival test): 970 µg/l	
Roux *et al.* 1993	moderate-hard synthetic (80-90 mg/l $CaCO_3$) (USEPA)		*D. pulex* • LC50 (48 h acute test) ~ 8 µg/l • NOEC-LOEC (reproduction test): 0.003-3 µg/l • α type toxicity expected, but hormesis also occurred for Cd, (for short exposure only)
Barber *et al.* 1994	hard (ASTM)	• O_2 consumption decreased for two clones • no effect upon weight gain under starvation (for both clones) • reduction in weight gain under intermediate food density not related to Cd concentration	
Allen *et al.* 1995	hard (ASTM)	• EC50 (24 h algal feeding test) ~ 2.634 µg/l cf. LC50 (48 h acute survival test) ~ 80 µg/l	
Juchelka and Snell 1995	NR		*C. dubia* • NOEC (1 h feeding test using fluorescent beads): 60 µg/l, cf. NOEC (reproduction test): 5 µg/l
Bitton *et al.* 1996	moderate-hard synthetic (USEPA)		*C. dubia* • EC50 (1 h CerioFAST feeding test): 54 µg/l, cf. LC50 (48 h acute survival test): 54 µg/l
Barata *et al.* 1998	range of soft to hard synthetic	• LC50 (48 h acute) range for 4 clones in softwater: 30.1 - 112 µg/l • hard water: 23.6 - 233 µg/l	
Taylor *et al.* 1998	hard (ASTM)	• LC50 (acute survival test) increase with presence of algae from 120 µg/l to >130 µg/l • feeding rate significantly lower 24 h after exposure with algae • cf. feeding rate significantly lower immediately after starved exposure (but less pronounced than when with food), however no significant difference after 24 h	

Reference	Water type	*D. magna*	Other species
C. Barata pers. comm.	hard (ASTM)	• EC50 (24 h algal feeding test): 2.1 µg/l • cf. EC50 (1 instar exposure - fecundity): 2.05 µg/l • cf. EC50 (1 instar exposure - brood mass): 1.52 µg/l • cf. EC50 (1 instar exposure - body mass): 6.11 µg/l • EC50 (2 instars exposure - 24 h algal feeding test): 0.86 µg/l • cf. EC50 (2 instars exposure - fecundity): 0.97 µg/l • cf. EC50 (1 instar - brood mass): 0.87 µg/l • only animals exposed to 10 µg/l died before starved animals	

* Not reported

1.6.4 Uranium

Uranium may occur in natural waters in 3 oxidation states: U^{4+}, UO_2^{+} and UO_2^{2+}. The speciation of uranium is relatively complex in oxidised fresh surface waters (pH 5-9), and changes in dominant species occur considerably particularly between pH 5-6. Natural DOM is an effective complexing agent (Moulin *et al.* 1992). It is generally considered that UO_2^{2+} is the form of uranium (VI) primarily responsible for eliciting a toxic response in aquatic organisms.

1.6.5 Additional note

Metal speciation models are a necessary component of calculating toxicity of metals to aquatic organisms (Witters 1998). However, the complexity and magnitude of such a study is beyond the scope of this research. Similarly, due to the complex pattern of uranium speciation, only preliminary trials were performed with uranium.

1.7 Aims

The aims of this research were:

1. To develop an ecotoxicological bioassay based on the feeding inhibition of *Moinodaphnia macleayi*, using algal cell density counts.
2. To compare the sensitivity of the feeding test against the currently used reproduction test, for the metals copper, cadmium and uranium.
3. To validate the developed feeding bioassay on mine release water.
4. To investigate any differences in mode of action copper and cadmium.

1.8 Null Hypotheses

This research tested the follow null hypotheses:

1. **There are no effects of copper, cadmium and uranium on *M. macleayi* feeding rate.**
2. **Feeding inhibition is not a more sensitive endpoint than reproduction for measuring toxic response to metal exposure.**
3. **There are no differences in feeding and reproductive responses following exposure to copper and cadmium.**

Chapter Two: Materials and Methods - General

2.1 Site Description

All research was performed at the Environmental Research Institute of the Supervising Scientist (*eriss*) Wetlands Laboratory, except for the initial month which was conducted at the Energy Resources of Australia (ERA) Ranger Uranium Mine Biological Testing Laboratory. Both laboratories are located at Jabiru East, NT. Research took place between July and December 1998.

Eriss is situated on the lease of Ranger Uranium Mines Pty. Ltd., an area completely surrounded by Kakadu National Park, NT. Magela Creek flows adjacent to the mine and runs into Magela Floodplain, an extensive wetland area of major ecological and cultural significance, and recognised with World Heritage Listing. It is part of *eriss*' statuatory role, to 'protect' these high conservation value wetlands from deterioration that might result from mining impacts (Section 36 of Environmental Protection Act 1978).

2.2 Diluent Water

Diluent waters used for culturing and testing were American Society of Testing and Materials (ASTM) soft synthetic water and filtered Magela Creek water (collected from Bowerbird Billabong, West Arnhem Land). Creek water was collected 5 times over the testing period (Appendix 1). Each day of each test, conductivity, dissolved oxygen and pH were measured for each treatment (Appendix 1). For ASTM soft water, mean (standard error (SE)) water parameters over all treatments were: conductivity:150 (10.) µS/cm; dissolved oxygen: 98 (0.6) %; and pH: 7.3 (0.3). For creek water, these were: conductivity: 18 (0.3) µS/cm; dissolved oxygen: 100 (2.0) %; and pH: 6.2 (0.1).

2.3 Chemical Analysis

Stock solutions were made according to a developed protocol (Appendix 2). Chemical analysis was performed on each treatment at the beginning and the end of each reproduction bioassay and at the beginning of each feeding test. Chemical analysis for the final day of the feeding test was discontinued

after establishing little change (<10 %) in metal concentration over test duration (Appendix 3). All analyses were performed at the Northern Territory University, Department of Applied Chemistry, using ICP-MS. Nominal and actual concentrations for all treatments for all tests are listed in Appendix 3. Mean (SE) differences between nominal and actual concentrations were: copper: 13 (0.8) %; cadmium: 8.7 (1.9) %; and uranium: 6.5 (1.0) %.

2.4 Testing Regime

Algal cell densities were measured using a Coulter® Multisizer II multichannel particle size analyser (sampling stand model IIA). Development of the feeding test protocols comprised a large proportion of the research (Figure 3). To avoid repetition, only deviations from the final test protocols (Appendix 4) are mentioned. Protocols were also developed for production of test animals (Appendix 5), taking Coulter counter counts (Appendix 6), calculation of cell density and feeding rate (Appendix 7) and performing concurrent feeding tests and reproduction tests (Appendix 8). Except where mentioned, all reproduction tests were performed according to protocols developed at *eriss* for *M. macleayi* (Figure 4) (Hyne *et al.* 1996), and based on the *D. magna* reproduction test (ASTM 1997).

The algal food source used was *Chlorella* sp., a single cell green alga cultured at *eriss* and used in *M. macleayi* culturing and reproduction bioassays (Hyne *et al.* 1996). Methods for culturing and harvesting algae, culturing test animals, and collection and storage of water, were adopted from *eriss* protocols (Hyne *et al.* 1996).

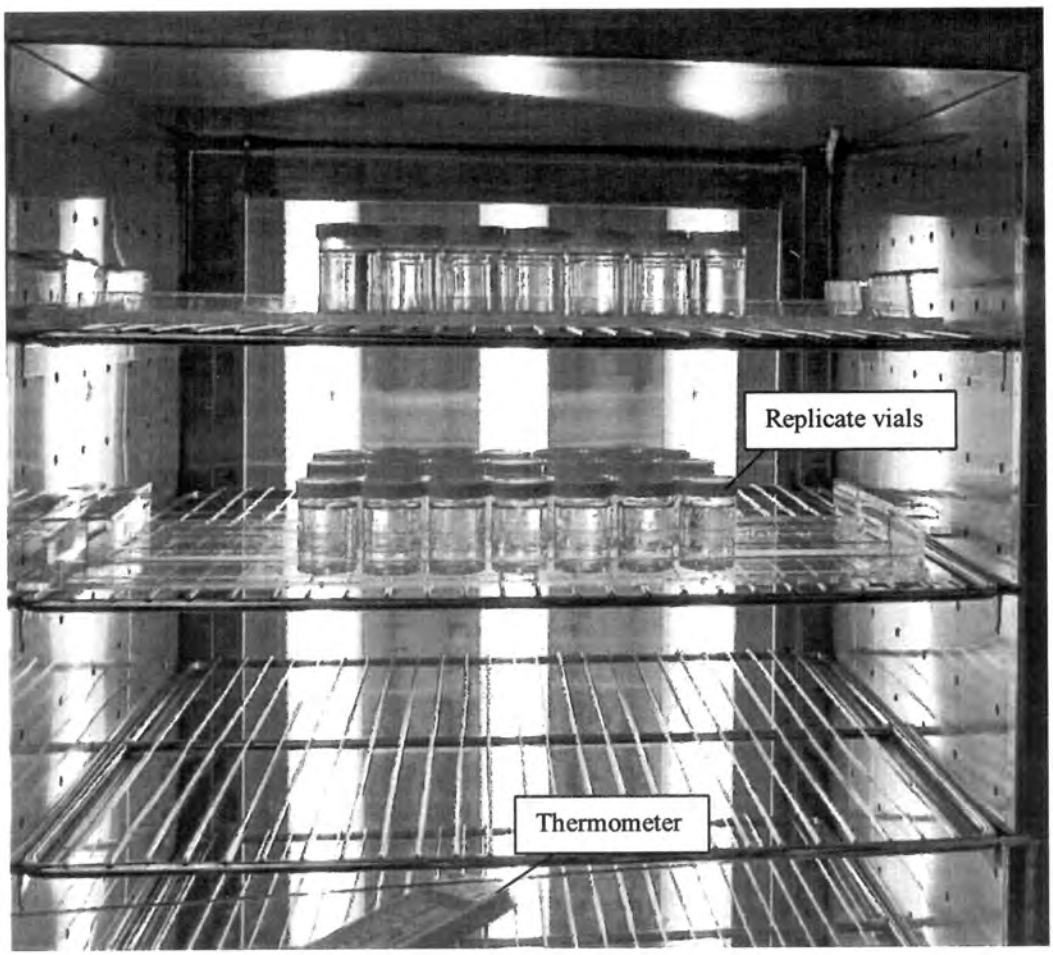

Figure 3. Photograph of *M. macleayi* feeding test exposure period.

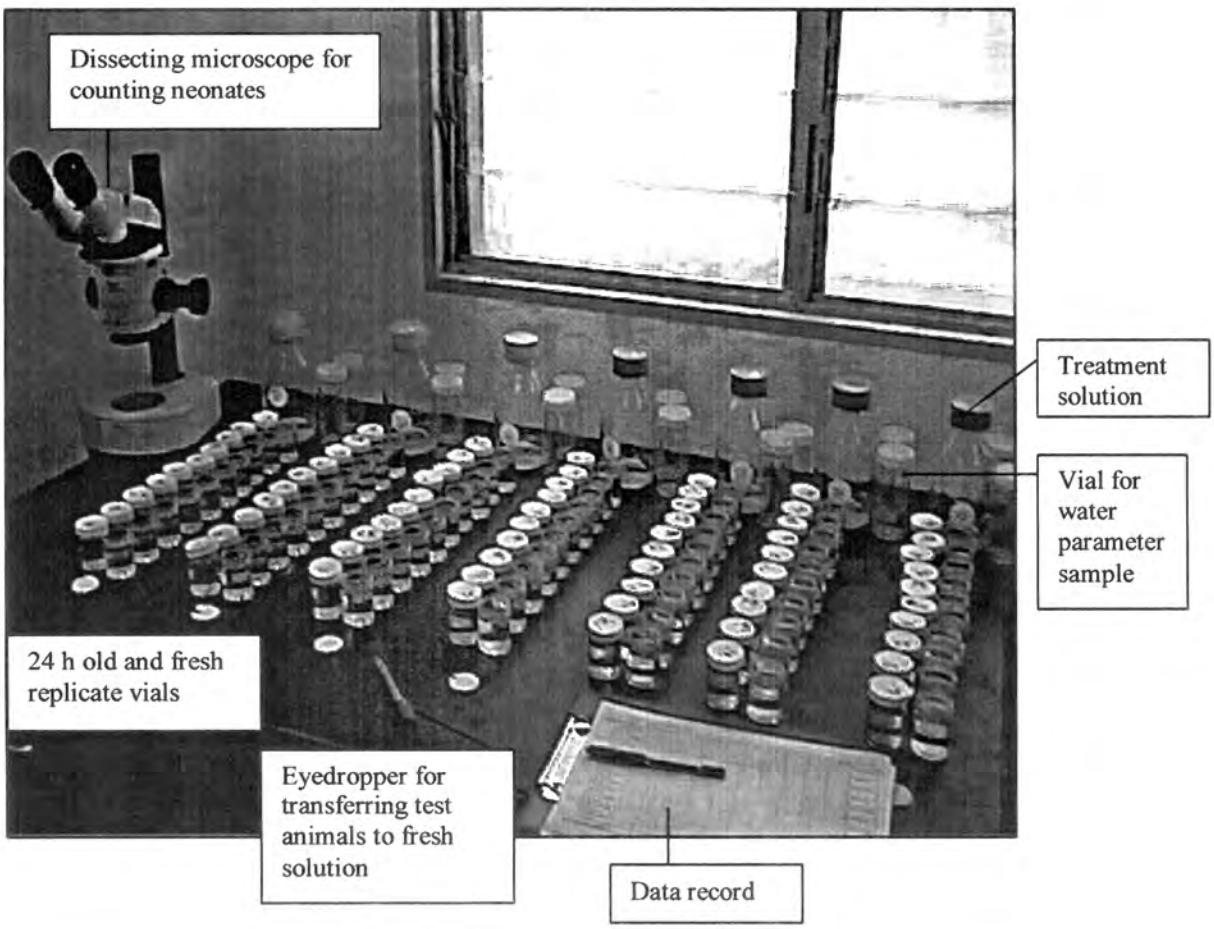

Figure 4. Photograph of *M. macleayi* reproduction test set-up.

2.5 Statistical Analysis

2.5.1 Choice of endpoint

There are 2 main procedures for determining statistical endpoints from chronic toxicity tests: hypothesis testing and point estimate techniques. Hypothesis testing usually determines the lowest test concentration that induces an effect statistically different from the control (LOEC), and in doing so, also the highest concentration which generates no significant effect (NOEC). Much of the toxicity testing reported in the literature has relied upon this approach as it is easy to apply and enables comparisons across differing testing regimes. However, such a method does not construct a dose-response relationship, and NOEC and LOEC values are largely dependent upon choice of test concentrations. In addition, the magnitude of the effect corresponding to the LOEC is dependent upon the statistical power of the test. Point estimate techniques derive a concentration estimated to effect *a priori* percent of test organisms. This method employs regression analysis models and hence assumes a continuous dose-response relationship. These estimates have the advantage of utilising all the information gathered as a part of the relationship. However, models vary widely, making comparisons difficult where different statistical tests or biological or statistical endpoints have been used. In the present research, both techniques have been used where deemed most appropriate.

2.5.2 Statistical analysis

The software package Statistica was used for statistical analysis during test development. Analysis involved comparisons of various treatments and factors using *t*-tests and 1-, 2-, and 3-way ANOVAs. Assumptions were tested using Bartlett's Test (for homogeneity of variances), visual assessment of residual plots and box and whisker plots. Data transformation was required for 1 test. Tukey's (*post hoc* pairwise comparison) Test was performed when a significant effect was detected.

The endpoints measured were feeding rate, in the feeding test, and average total offspring number, in the reproduction test. In addition, average brood size was calculated in the reproduction test. This was a measure of average total offspring for only those test animals that survived to produce 3 broods within the test period. Therefore, this response eliminates effects from delayed reproduction and adult mortality. By

comparing effects to average total offspring and average brood size, more specific reproductive responses could be identified.

Toxicity test analyses (both hypothesis testing and point estimate techniques) were performed a using recommended USEPA flowchart method on ToxCalc^{TM} software. For hypothesis testing, this involved checking assumptions with Bartlett's Test (for homogeneity of variance) as well as Shapiro-Wilk's Test (for normal distribution). Residual plots and box and whisker plots were also assessed. Where data conformed to assumptions and a significant effect detected, Dunnet's (*post hoc* pairwise comparison) Test was performed to establish NOECs and LOECs. Transformations were attempted on data sets which failed assumption tests. However this did not improve normality and variance sufficiently for any test. For these cases, non-parametric testing (Steels Many-One Rank Test) was performed.

Two-tailed hypothesis testing was chosen due to the possibility of an increase (in feeding rate or offspring number) response, relative to the controls. The α level was set at 0.05 for all analyses.

Using regression analysis, concentrations causing a 5 and 20 % effect in test organisms relative to the control (EC5 and EC20, respectively), with 95 % confidence intervals (CI), were calculated. These are the point estimates most commonly cited in the literature as they are considered comparable to NOECs and LOECs. Probit analysis was used to calculate the concentration lethal to 50 % of the test organisms relative to the control in a given time (LC50) (with 95 % CI) for some tests where mortality was high. Standard Error of the Difference tests (Sprague and Fogels 1977) were performed to compare point estimates between tests.

2.5.3 Reporting results

Actual concentrations have been reported for all results except where trends within single tests were analysed, or where data has been presented graphically.

Chapter Three: Feeding Test Development

3.1 Introduction

The objective of the initial phase of test development was to establish protocols enabling reliable detection of feeding rate in control animals. Other main considerations were: 1) environmental realism, 2) health of animals, and 3) uniformity with other cladoceran bioassay protocols. In particular, test variables employed in the *M. macleayi* reproduction bioassay (Hyne *et al.* 1996) were used as much as possible. This was both to adopt optimised culturing and testing methodologies for *M. macleayi*, and to enable direct comparisons of feeding and reproductive responses.

Certain biotic and abiotic factors are known to modify the results of toxicity tests (Schindler 1968; Cowgill 1987; Holdway 1992a, b). The influence of the following potential modifying factors were chosen for investigation: algal density, animal density, form of algae (fresh or frozen), use of food additives, water type, light regime, clonal variation, sample volume size and Coulter counter protocols. Other modifying factors considered important were temperature, age of test animal and test duration. These factors were not tested but levels were decided upon prior to testing, after review of the literature and logistical consideration (Appendices 9-11).

3.2 Testing of Modifying Factors

3.2.1 Animal and algal density

Animal and algal densities had to be established at levels allowing clear and reliable detection of feeding inhibition. The feeding depression should be low enough to avoid the possibility of food limitation, and high enough to obtain a dose response relationship. An ideal feeding depression was considered *a priori* to be 20-30 % of the initial cell density.

Aim. To determine an animal and algal density which would yield a feeding depression of approximately 20-30 % of initial cell density, with relatively low variance.

Null Hypothesis. **There are no effects of animal and algal density on feeding depression.**

Methods. Six treatments were used to assess 2 algal densities and 3 animal densities. These were algal density of 2×10^5 cells/ml, with animal densities of 1, 2 and 4 individuals per 30 ml sample, and algal density of 5×10^5 cells/ml, with animal densities of 2, 4 and 10 individuals per 30 ml sample. Each treatment comprised 3 replicates and 2 blanks (no animals). The water used was ASTM soft synthetic water, temperature ranged from 27-31 °C and the test duration was 24 h.

Results. There was no significant difference in feeding rate between treatments. The only 2 treatments to provide a mean (SE) feeding depression greater than 20 % of the initial cell density were 4 animals in 2×10^5 cells/ml algal density (24 (13.6) %) and 10 animals in 5×10^5 cells/ml algal density (30. (6.6) %).

Conclusion. Performing the feeding test with 10 animals per replicate would require high culturing and handling demands. In addition, the reproduction bioassay is performed with an algal density of 2×10^5 cells/ml. Hence this algal density, with an animal density of 4 animals per 30 ml sample, was chosen for all further testing.

3.2.2 Algal form and light regime

Significant algal growth over the test duration was noted for several initial tests performed with a food source of fresh *Chlorella* sp. and a 12/12 light/dark light regime in a constant temperature incubator. Alternative algae and light conditions considered were: using frozen (but thawed) algae and retaining the 12/12 light/dark regime, or using fresh algae in a dark incubator. The original 12/12 light/dark regime was employed to maintain consistency with the reproduction bioassay, however, it has been reported that cladoceran feeding is higher and more consistent in dark conditions (Haney 1985).

Two tests were performed to investigate the effects of algal type and light regime on a) change in algal cell counts; b) cladoceran feeding rates; and c) toxicity of copper to *M. macleayi.*

Test 1: The effect of copper and light regime on fresh and frozen algae over time

Aim. To establish effects of copper, light, algal type (fresh or frozen), and their interactions, on algae cell counts

Null Hypothesis. **There are no effects of copper concentration, light regime, algal type, and/or their interactions, on algal cell counts over time.**

Method. Three factor levels were: algal type (fresh and frozen (but thawed) algae); light regime (dark and light/dark (12/12)); and copper concentration (0 (control), 10 and 60 µg/l). Test water was ASTM soft synthetic water with a seaweed extract food additive (SEA) (see Chapter 4). At the end of the 20 h exposure period (t_{20}), 3 replicates were counted for each of the 12 treatments. *t*-tests were used to determine if t_{20} counts were significantly different to counts taken at the beginning of the test (t_0). In addition, for each treatment, changes in algal cell counts (from t_0 to t_{20}) were measured and a 3-way ANOVA performed on the square-root transformed data to determine if any factor (or any interaction) caused a significant change in algal cell counts in comparison with other treatments.

Results. The following treatments increased significantly over time: fresh algae in the light/dark at 0 and 10 µg/l Cu. The following treatments decreased significantly over time: fresh algae in the dark, frozen algae in the dark at concentration 60 µg/l Cu, and frozen algae in the light/dark at 10 and 60 µg/l Cu. There was a significant effect of each of the 3 factors, as well as each of the interactions, upon change in cell counts over time (Appendix 13). The following interpretations can be made from the *post hoc* 3-way interaction table: light had a (positive) effect upon fresh (but not frozen) algal counts; and copper had a (negative) effect upon both fresh and frozen algal counts. The only algal-light regime combination where copper concentration had no significant effect on algal counts was fresh algae in the dark.

Conclusion. The combination of fresh algae in a light/dark incubator could not be used in a feeding test due to significant algal growth over time. The negative effect of copper on algal cell counts was possibly as a result of lysis of the algal cell. However, for fresh algae in the dark, this reduction in cell count was not significant, and hence this combination provided the most appropriate testing regime. To consolidate this protocol however, the effect of each regime upon cladoceran feeding rate was assessed.

Test 2: The effect of algal type and light regime upon feeding rate

Aim. To measure the effect of 3 feeding regimes upon *M. macleayi* feeding rate: frozen algae under dark and light (12/12) light regimes, and fresh algae in the dark.

Null Hypothesis. **There are no effects of feeding regime on *M. macleayi* feeding rate.**

Methods. Three treatments were trialed: frozen (but thawed) algae in both dark and light/dark (12/12) light regimes, and fresh algae in the dark. ASTM soft water was used and SEA added to all treatments.

Results. High mortality was observed in all treatments: fresh algae in the dark (15 %), frozen algae in the dark (20 %) and frozen algae in the light/dark (40 %). There was an effect of treatment on feeding rate. The coefficient of variation (CV) was lowest for the treatment fresh algae in the dark (0.4 %, compared with 8.5 and 3.4 % for frozen algae in dark and light/dark treatments, respectively).

Conclusion. In corroboration with the previous test, the feeding regime of fresh algae in dark conditions was found to be most appropriate, this time on the grounds of lower animal mortality and within treatment variance. The lower variance may have been partly due to the lower mortality, and hence higher sample size. Retaining fresh algae, but changing to a dark (light-) regime also appeared a smaller deviation from both reproduction test protocols and environmental realism than maintaining 12/12 light/dark conditions and changing to a frozen algae food source.

3.2.3 Water type

ASTM soft water was initially trialed for culturing and testing. Synthetic water was chosen to enable higher replicability, direct comparisons with other research and a broader application of the developed bioassay. Soft water was chosen as being more chemically similar to the local water type. However, animal survival in the cultures was below OECD standards (≥80 %) (OECD 87), even with the addition of a food additive (SEA). High mortality (Van Leeuwin *et al.* 1985) and reduced fecundity (Baird *et al.* 1989) in synthetic water cultures have been reported for other cladoceran species, and observed for *M. macleayi* (R. van Dam pers. comm.). In addition, 4 feeding tests run with ASTM soft water provided unsatisfactory results; problems included high mortality and response variance in all treatments (Appendix 14).

Local creek water from Magela Creek, filtered at 10 µm (Whatman no. 1 filter), is used successfully for the *M. macleayi* reproduction bioassay as well as for culturing test animals (Hyne *et al.* 1996). In order to increase culture and test animal survival, and concur with the reproduction bioassay protocols, the use of filtered creek water in the feeding test was trialed. However, the increased particulate organic matter (POM) and DOM load of the creek water, in comparison with the synthetic water, introduced the following potential problems: a) POM would provide a significant alternative food source for the animals, significantly reducing the measured feeding depressions, b) background 'noise' in the Coulter cell counts would impair the reliability of algal cell counts, and c) POM and DOM would alter the bioavailability of the toxicant, affecting the animals' sensitivity to the toxicant. Three tests were performed to investigate these issues.

Test 1: The effect of water type on Coulter cell counts

Aims. To establish if:

1. Background 'noise' forms a significantly larger component of filtered creek water cell counts, than ASTM water.
2. To observe any visual differences in algal 'peaks' between the 2 water types.

Null Hypotheses.

1. There are no effects of water type on background counts.

2. There are no visible differences in algal 'peaks' between water types.

Methods. Two water types were used: ASTM soft water, and filtered (10 µm) Magela Creek water. Water types were tested with and without algae. SEA was added to all treatments. One full range (in order to observe algal 'peak') and 4 narrow range counts were taken at t_0 for all 4 treatments (water type (×2); with and without algae (2)). At t_{20}, 3 replicates for each treatment were counted in the same manner. For t_0 and t_{20}, percent-algae component (of narrow range count) was calculated for each water type (100 × (algae count - without algae count) / algae count). A *t*-test was used to determine a difference between the t_{20} treatment counts.

Results. At t_0, algae comprised 94 % of both ASTM and creek water narrow range counts. At t_{20}, the mean (SE) percent-algae component remained stable for the ASTM water (93 (0.4) %) but had decreased 10 % for creek water (84 (0.3) %). There was an effect of water type on background cell count at t_{20}. There was no significant visual difference in narrow algal 'peaks' for the 2 water types.

Conclusion. The increase in background matter over time in the creek water introduced potential problems. Additional POM in the water could create an alternative food source for test animals, and could affect the complexing of any toxicant tested. However, using creek water would increase the tests environmental realism, as well as concordance with the reproduction bioassay. Despite the higher POM load in the t_{20} creek counts, within treatment variability remained low, therefore detection of feeding inhibition should potentially be measured with similar accuracy to tests with synthetic water.

Test 2: The effect of water type on feeding rate and copper toxicity

Aim. To determine if water type (ASTM soft and filtered creek water) affects *M. macleayi* feeding rate and sensitivity to copper.

Null Hypotheses. **There are no effects of water type on the feeding rate of *M. macleayi*, and/or the sensitivity of *M. macleayi* to copper.**

Methods. Treatments comprised 2 water types: ASTM soft water and filtered (10 µm) creek water; and 3 levels of copper concentration: 0 (control), 30 and 60 µg/l. SEA was added to all treatments. Parents of test animals were cultured in ASTM water. After release of second brood (test animals), half of the neonates were transferred to 50-50 % ASTM-creek water for 1 d and then to 100 % creek water for 2 d. At t_0, 2 replicates for each treatment were counted, and at t_{20}, 5 replicates along with 2 blanks were counted. A 2-way ANOVA was used to test for an effect of water type, copper concentration and their interaction, upon feeding rate.

Results. At t_{20}, mortality in copper concentrations 0, 30 and 60 µg/l was 5, 5 and 55 % in the ASTM water, and 0, 0 and 100 % in the creek water. There was no significant effect of water type, copper (for concentrations 0 and 30 µg/l) or their interaction upon feeding rate. For copper treatments 0 and 30 µg/l,

the feeding rate coefficients of variation were lower in creek water than in ASTM water (4.8 and 9.9 %, compared with 34 and 18 %, respectively).

Conclusion. There was no effect of water type on algal feeding rate. It was also noted that test animals raised in creek water released larger neonates, and up to 12 h earlier than animals raised in ASTM water. This observation, as well as the lower within-treatment variance, corroborates previous reports that animals raised in natural water are considerably more healthy than those raised in ASTM water. Using mortality as an endpoint, toxicant sensitivity was increased in the creek water treatments. The increased health and toxicant sensitivity of animals in filtered creek water provide support for this water type to be used in the feeding test.

Test 3: The effect of filter type (10 and 2.5 μm) upon creek water Coulter counts

Aim. To establish if filter type affects percentage of background 'noise' in creek water Coulter counts.

*Null Hypothesis.***There are no effects of water filter size (10 and 2.5 μm) on the proportion of background 'noise' in algal cell counts.**

Methods. As described in *Test 1*, except that 3 water types were used: ASTM soft water, creek water filtered at 10 μm and creek water filtered at 2.5 μm (Whatman filter no.42). ASTM soft water was used to corroborate the results of the previous test (i.e. *Test 1*). Within treatment variance was investigated by calculating the percent-algae composition for each of the 3 'with-algae' replicates with each of the 3 'without-algae' replicates, producing a total of 9 cell counts for each treatment.

Results. At t_0, algae comprised 94, 97 and 97 % of cell counts for ASTM water, creek water (10 μm filter) and creek water (2.5 μm filter), respectively. At t_{20}, these mean (SE) percentages were 97 (0.4), 92 (0.8) and 95 (0.2) %.

Conclusion. The percent-algae compositions were slightly higher when the creek water was filtered at 2.5 μm in comparison to at 10 μm. In addition, the change in percent-algae composition over time was least for this water type, and with the lowest variance. Hence all future testing was performed using 2.5 μm filtered creek water.

3.2.4 Other factors

Single tests were performed to establish the most appropriate sample volume size (Appendix 15) and Coulter counter dilution factor (Appendix 16). Clonal variation has also been recognised as a factor that can significantly affect response results in toxicity tests. Due to time constraints, it was not possible to conduct a more thorough investigation of this area of research. However, a pilot study was conducted with the objective of aiding future cladoceran toxicity test research at *eriss* (Appendix 17). The effect of diet required extensive investigation and is discussed in chapter 4.

Chapter Four: The Effect of Diet

4.1 Introduction

It has been proposed that contaminated food is a major route of metal uptake in freshwater organisms (Campbell 1995). This is most likely due to the free metal ions adsorbing to the algal cell membrane, and then becoming unbound by enzymes and acids in the gut of the animal (Taylor *et al.* 1998). The presence of DOM is also known to affect the toxicity of metals to freshwater organisms, usually by reducing the metal's bioavailability (Campbell 1995). It is thought that this is due to DOM-metal complexes reducing the concentration of the free metal ion in solution. Therefore, food source quantity and quality, as well as additional organic matter will potentially affect metal toxicity in laboratory bioassays, as well as in the field.

In the present study, it was deemed important to find a food source that would not affect algal cell counts in the feeding bioassay, but would enable adequate survival (≥80 %) and fecundity (≥20 neonates per adult) in the reproductive bioassay (OECD 1987). A dissolved organic additive of seaweed extract (SEA) (Appendix 12) has been used successfully for culturing *D. magna* (Baird *et al.* 1989), hence was trialed for use in *M. macleayi* culturing and bioassays.

Three issues required investigation: a) whether the addition of the SEA would significantly affect the algal cell counts of the algal suspension, b) whether adequate survival would be achieved in the reproduction bioassay when using a diet of *Chlorella* sp. and SEA, and c) whether the addition of the SEA would significantly affect the sensitivity of *M. macleayi* to the toxicant.

4.2 Testing Procedure

4.2.1 The effect of a dissolved food additive on Coulter cell counts

Aim. To measure the effect of SEA on algal cell counts.

Null Hypothesis. **There are no effects of SEA upon algal cell counts.**

Method. One treatment comprised the addition of SEA to the control algal suspension in ASTM soft water. At t_0, a single set of cell counts were taken for both the control and treatment, and at t_{24}, 5 samples of each were counted. A *t*-test was performed to compare cell densities between the control and treatment at t_{24}.

Results. There was no significant difference between control and treatment cell counts at t_{24}.

Conclusion. Algal cell counts were not affected by the addition of the SEA. Therefore, if sufficient survival can be achieved in the reproduction bioassay, SEA could potentially be a suitable food additive to use in both bioassays. This would enable direct comparison of feeding and reproductive responses to chemical concentrations.

4.2.2 The use of SEA as a food additive in the M. macleayi reproduction bioassay

Aim. To investigate the use of SEA as a food additive in the *M. macleayi* reproduction bioassay (with copper exposure), and in particular establish whether adequate control survival and fecundity can be achieved.

Null Hypothesis. **The use of SEA as a food additive (instead of FFV) has no effects on control survival and fecundity.**

Methods. Four reproduction bioassays were performed according to standard protocols with the exception that SEA was used as a food additive, instead of FFV. Copper concentrations were: *Test 1*: 0 (control), 10, 15, 20, 30, 40 and 50 µg/l; *Test 2*: 0 (control), 10, 20, 25 and 30 µg/l; and *Test 3* and *4*: 0 (control), 10, 20, 25, 30 and 35 µg/l. *Tests 2-4* also employed a second control treatment using FFV instead of SEA as a food additive.

Results. After 5 d, there was adequate survival in the SEA controls in only 1 of the 5 tests (Table 3). Offspring number was not adequate in 1 of the tests, and in 2 others was not significantly above the lower threshold. There was adequate survival and offspring number in FFV controls in 2 of the 3 tests. In the test where sufficient survival was not obtained, offspring number was not significantly above the lower threshold.

Conclusion. FFV control animals showed higher survival and fecundity than SEA control animals in 2 of 3 of the tests. The 1 test where FFV controls faired poorly was considered an anomaly, as test (control) and

Table 3. Day 5 mortality and mean (SE) total offspring number for cladocerans fed diets of fermented cichlid food (FFV) and seaweed extract additive (SEA).

	Mortality (%)		**Mean (SE) total offspring number**	
Test	**FFV**	**SEA**	**FFV**	**SEA**
Test 1	-	50	-	19 (2.0)
Test 2	20	40	29 (3.1)	22 (0.8)
Test 3	80	20	21 (5.3)	22 (4.0)
Test 4	0	30	31 (1.9)	27 (2.1)

cultured animals have consistently survived and reproduced adequately in this medium (R. van Dam pers. comm.). Due to poor survival and reproduction in SEA control animals, original reproduction bioassay protocols were resumed.

4.2.3 Effect of diet on M. macleayi sensitivity to toxicants

Test 1: The effect of 2 different diets on the sensitivity of M. macleayi to copper

Aim. To measure the sensitivity of *M. macleayi* to copper using 2 different diets: *Chlorella* sp. and *Chlorella* sp. with SEA.

Null Hypothesis. **SEA food additive has no effects on the sensitivity of *M. macleayi* to copper.**

Method. Two diets were tested: algae-only (ALGAE) and algae.and seaweed extract (SEA); and 5 copper concentrations were used: 0 (control), 5, 10, 20 and 40 µg/l. Four replicates were counted for each treatment at t_{20}. A 2-way ANOVA was performed to test for an effect of diet, copper concentration, and their interaction.

Results. Mortality was 100 % in ALGAE treatments at 20 and 40 µg/l Cu, and 15 % in the SEA treatment at 5 µg/l Cu. For this reason, a 2-way ANOVA was only performed for treatments 0, 5 and 10 µg/l Cu. Feeding rate was not significantly affected by copper concentration, food type, or their interaction.

Conclusion. There was no significant feeding effect detected in any treatment. However, the mortality data suggested that the sensitivity of the animals to copper exposure was increased when an algae-only diet was used.

Test 2: The effect of 3 different diets on the sensitivity of M. macleayi to cadmium

Aim. To measure the sensitivity of *M. macleayi* to cadmium using 3 different diets: algae-only, algae with SEA, and algae with FFV.

Null Hypothesis. **Diet has no effects on the sensitivity of *M. macleayi* to cadmium.**

Method. Three diets were tested: algae-only (ALGAE), algae and seaweed extract (SEA), and algae and fermented cichlid food (FFV); and 4 cadmium concentrations were used: 0 (control), 5, 10 and 20 µg/l.

Three replicates were counted for each treatment at t_{20}. A 2-way ANOVA was used to test for an effect of diet, cadmium concentration, and their interaction.

Results. There was a significant effect of cadmium concentration, diet, and their interaction. The animals were most sensitive to cadmium when feeding on diets ALGAE and SEA (Table 4). At the 2 lowest concentrations, feeding depression was highest in the ALGAE treatments. There was significantly higher feeding in the ALGAE control than the SEA and FFV controls, which were not significantly different from each other.

Conclusion. Lower algal feeding rates were measured in the SEA and FFV controls (in comparison with ALGAE controls) suggesting that the test animals were feeding significantly on the alternative food sources which lay outside the algal 'peak'. As all treatments were compared with a control of the same diet, this should not affect measurement of feeding effects unless the test animals can 'taste' the contaminated algae at higher toxicant concentrations, and alter the proportion of algae in their diets. ALGAE treatments yielded the equal lowest LOEC, and highest feeding depression at lower concentrations. A sudden reduction in feeding was recorded in the higher concentrations of the FFV treatment. However, patterns detected at the higher concentrations are of less ecological relevance than effects detected at trace levels. In addition, a more simplified diet would probably attenuate metal speciation. Thus, a diet of algae-only was considered the most appropriate food source.

4.2.4 Further evidence of the effect of diet on M. macleayi sensitivity to toxicants

In addition to the above tests that directly tested the effect of different diets on sensitivity to toxicants, feeding tests were performed separately with each diet for each toxicant. None of these tests were run concurrently, however, their results can be used to corroborate findings from tests where different diets were tested simultaneously.

Aim. To compare results of copper feeding and reproduction tests, and cadmium feeding tests, where different diets have been used.

Null Hypothesis. **There are no effects of diet on the results of copper feeding and reproduction tests, and cadmium feeding tests.**

Table 4. Mean (SE) control feeding rates and percent feeding depressions for cadmium-exposed cladocerans fed diets of algae-only (ALGAE), and algae with fermented cichlid food (FFV) or seaweed extract additive (SEA). Asterisk (*) indicate significant difference from controls.

Diet	**Control feeding rate (cells/animal/h)**	**Feeding depression (%) at 5 µg/l Cd**	**Feeding depression (%) at 10 µg/l Cd**	**Feeding depression (%) at 20 µg/l Cd**
ALGAE	4.2×10^4 (962)	16 (0.9)	35 (2.9) *	51 (1.7) *
SEA	3.5×10^4 (932)	14 (2.4)	24 (3.4) *	50 (9.9) *
FFV	2.8×10^4 (375)	13 (6.0)	21 (5.9)	74 (0.4) *

Methods.

Copper Feeding Tests. Four copper feeding tests were performed: 3 with a SEA diet. (Chapter 5.2.1 *Tests 1-3*), and 1 with an ALGAE diet (Chapter 5.2.1. *Test 5*). Mortality data was not amenable to probit analysis, however interpretations were made from the raw data.

Copper Reproduction Tests. Five copper reproduction tests were performed: 4 with a SEA diet (Chapter 4.2.2. *Tests 1-4*), and 1 a FFV diet (Chapter 5.2.1. *Test 5*). Probit analysis was used to compare survival, and regression analysis to compare reproduction, between tests. Standard Error of the Difference tests were used to compare point estimates.

Cadmium Feeding Tests. Four feeding tests were performed with cadmium; 2 with a SEA diet (Chapter 5.2.2. *Tests 2-3*) and 2 with an ALGAE diet (Chapter 5.2.2. *Tests 4-5*). Standard Error of the Difference tests were performed on the EC5s and EC15s (as EC20s were not available for all tests) to detect significant differences between these test point estimates. NOECs and LOECs were determined for all tests.

Results.

Copper Feeding Tests. Feeding inhibition was not a sensitive endpoint for copper (Chapter 5). In the 3 SEA feeding tests, mortality ranged between 0-55 % at 30 µg/l Cu, whereas in the algae-only feeding test, mortality was 100 % at 17 µg/l copper (Table 5).

Copper Reproduction Tests. The 4 SEA reproduction tests were not valid, due to low (<80 % before release of brood 3) control survival (Table 3). However, effects can be measured from total offspring data over a 5 d period. Results do not suggest a significant effect of diet on the results of these tests (Figure 5).

Cadmium Feeding Tests. Results from point estimate techniques suggest a possible effect of diet on the results of cadmium feeding tests (Figure 6). When hypothesis testing is applied, the effect of diet on feeding test results is significant (Figure 7).

Conclusion.

Mortality data from the copper feeding tests, and results from the cadmium feeding tests corroborate the hypothesis that sensitivity to copper and cadmium is reduced when organic matter is added to the algal

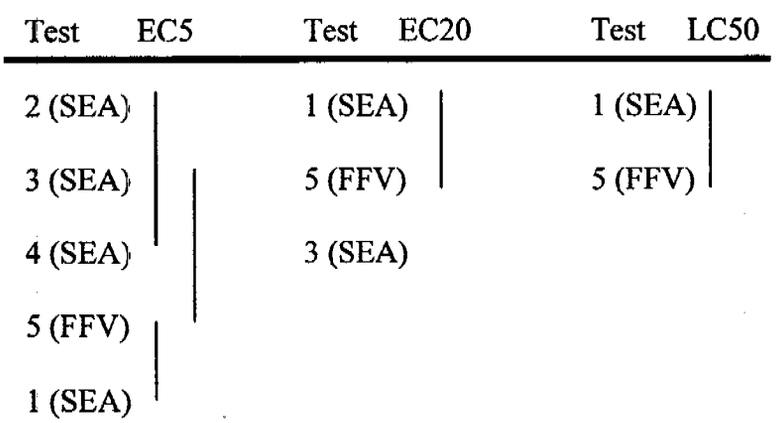

Figure 5. Similarities in point estimates for copper reproduction tests using diets of SEA and FFV food additives. Lines indicate no significant difference.

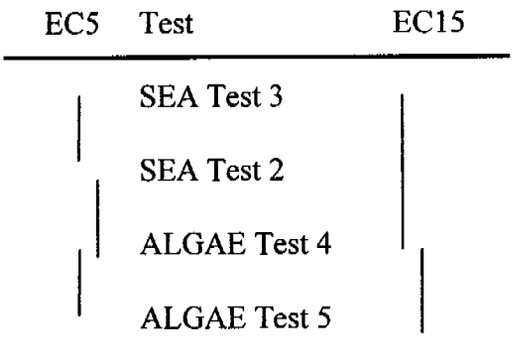

Figure 6. Similarities in point estimates for cadmium feeding tests using diets of algae-only (ALGAE) and algae with seaweed extract (SEA). Lines indicate no significant difference.

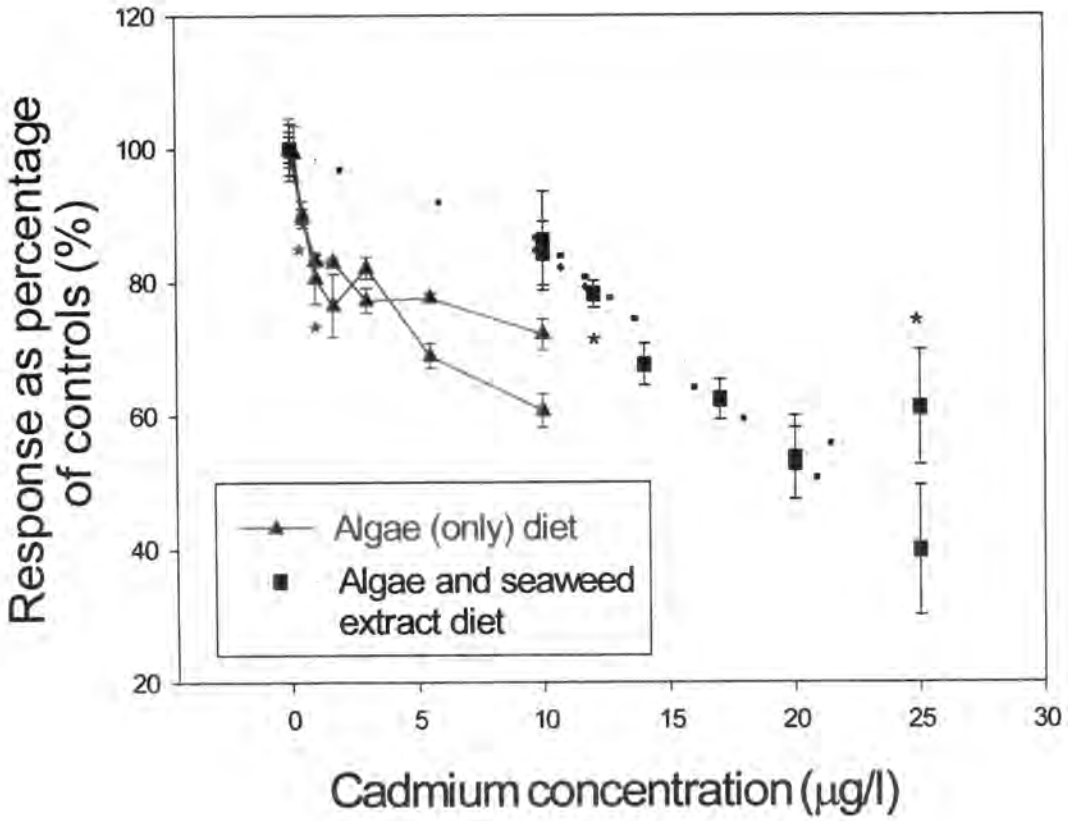

Figure 7. Feeding response as percentage of controls (mean (SE)) for cadmium exposure, when using diets of algae (only) and algae and seaweed extract. Asterisk (*) indicates lowest concentration that was significantly different from controls (LOEC).

food source. This inference is strongly supported by the cadmium feeding test results yielded from hypothesis testing, and less so by results from the point estimates. The discrepancy between results for these 2 techniques raises the importance of constructing a test design appropriate to *a priori* objectives. The primary objective in the current tests was to detect statistically significant feeding inhibition within a single test. In order to construct a strong dose-function with narrow confidence limits, the test design would have to be reassessed.

4.3 Discussion

Diet was a significant modifying factor in the present cladoceran feeding trials. Sensitivity to both cadmium and copper were lowered when animals were provided an organic additive in addition to the algal food source.

Different diets can influence the sensitivity of animals to toxicants in several ways. Firstly, poor nutrition may induce stress in an animal population leading to a lowering of resistance to toxicant exposure (Cowgill 1987; Lanno *et al.* 1989). Effects may include an altered metabolic rate, and hence the uptake, metabolism and depuration rate of a toxicant, and may even move the organism from a maintenance, to a catabolic or an anabolic physiological state (Lanno *et al.* 1989). Taylor *et al.* (1998) found that over a 4 h period in clean algae, animals which had been starved for 24 h had significantly lower feeding rates than animals which had previously been fed. Furthermore, several studies have found that animals raised on algal diets (including with food additives) have a reduced sensitivity to toxicants than animals raised on synthetic diets (Cowgill 1987; Rippon 1993).

The use of FFV reduced the toxicity of cadmium in the feeding test, in comparison to SEA. However, there appeared no significant effect of FFV in comparison to the SEA additive upon copper toxicity in the reproduction test. This effect could be due to experimental error, metal type, test duration or biological parameter.

In the mixed diet feeding tests (Chapter 4.2.3 *Tests 1-2*), animals from all treatments were raised on identical diets. Therefore, it is improbable that nutritional status alone could be responsible for the differences between animal sensitivity to toxicants. A more likely alternative is that the additional organic matter in the suspension affected toxicity by altering the bioavailability of the toxicant (Campbell 1995).

Trace metals are known to rapidly bind to the surface of algal cells within 10 min, accompanied by a slower uptake into the cytoplasm of the cell (Gipps and Coller 1980; Ting *et al.* 1989). Taylor *et al.* (1998) performed 20 min tests exposing *Chlorella vulgaris* suspensions of 5×10^5 and 1×10^6 cells/ml to cadmium. They found that there was a strong increase in cadmium mass binding to individual algal cells with increasing cadmium until concentrations exceeded 100 µg/l. Hence, at low concentrations (i.e. concentrations used in the present study), additional DOM may reduce the mass of cadmium binding to algal cells over short time periods by complexing available cadmium ions.

Research has also found that quantity of food is another test variable with the potential to confound bioassay results. Chandini (1989) found that high ambient food levels greatly reduced toxic effects of cadmium in *D. carinata* for parameters of mortality, growth and reproduction. Taylor *et al.* (1998) also reported increased LC50s (hence, reduced toxicity) for *D. magna* exposed to cadmium when food was introduced. These responses may not only be due to the organism's improved physiological state, but also as a consequence of the additional POM (food) reducing the bioavailability of the metal.

Buikema Jr. (1980) suggested that the unrealistically high food densities used in toxicity bioassays are likely to provide under- or over- protective results. Lower food densities are generally encountered in nature, possibly resulting in reduced sensitivities to toxicant exposure. Tessier *et al.* (1983) observed that storage quantity in young daphnids depended upon parental feeding success, with offspring of well-fed adults surviving for twice as long when starved than offspring of starved adults. Interestingly, Enserink *et al.* (1993) found that at low food rations, *D. magna* produced smaller broods of larger neonates, possibly providing the neonates with increased food reserves, and hence increasing their survival possibilities.

It is important when developing toxicity test feeding protocols, to choose feeding regimes according to *a priori* objectives of the test, and with acknowledgment of any shortcomings that this regime may present. Objectives of the present testing regime required that the food type chosen a) not affect Coulter counts, b) yield adequate survival and reproduction, and c) engender maximum sensitivity of the animal to the toxicant. Unfortunately, no single diet fulfilled all requirements. The only food type found to be suitable in the reproduction bioassay was *Chlorella* sp. with FFV, supporting the findings of Hyne *et al.* (1993) and R. van Dam (pers. comm.). However, this diet did not provide maximum sensitivity in the feeding bioassay. At this stage of feeding test development, the importance of optimising the test's sensitivity and maximising the simplicity of the feeding regime warranted a departure from the reproduction test protocols for diet. This divergence impedes drawing direct parallels between feeding inhibition and reproductive effects for toxicant concentrations.

Chapter Five: Feeding Test Application

5.1 Introduction

The second phase of feeding test development involved measuring feeding responses from toxicant exposure. Aims were: a) to compare the relative sensitivity of the feeding test with the reproduction bioassay for copper and cadmium; b) to compare feeding and reproductive responses for *M. macleayi* when exposed to copper and cadmium; c) to measure feeding inhibition for *M. macleayi* exposed to uranium; d) to compare feeding and reproductive responses for copper and cadmium, and e) to validate the feeding test by assessing multi-toxicant mine release water, and compare the sensitivity of the test with the reproduction bioassay.

5.2 Materials and Methods

5.2.1 Copper

Tests 1 – 3: Comparison of feeding and reproduction bioassays: a) using seaweed extract additive.

Three feeding tests were performed using filtered creek water (first test using a 10 µm filter, and the following 2 using a 2.5 µm filter) and SEA as a food additive. Copper concentrations used were: *Test 1*: 0 (control), 10, 15, 20, 30, 40 and 50 µg/l; *Test 2*: 0 (control), 10, 20, 25 and 30 µg/l; and *Test 3*: 0 (control), 10, 20, 25, 30 and 35 µg/l. Only 3 replicates were used for each treatment in *Test 2*. Each test was run concurrently with a reproduction bioassay (using SEA as a food additive) (Chapter 4.2.2 *Tests 1-3*, respectively).

Test 4: Comparison of feeding and reproduction bioassays: b) feeding test with pre-exposure.

Feeding inhibition was not detected over 20 h in copper exposure tests (Chapter 5.3.1). Therefore, it was investigated whether longer exposure periods would induce feeding inhibition. Copper concentrations used were 0 (control), 10, 20, 25, 30 and 35 µg/l. Twenty-four hours before commencement of the test (when test animals were carrying brood 1, non-eyed-neonates), 6 Petri dishes were prepared containing 300 ml of

each test treatment solution (including control). Two animals were removed from the test animal culturing bowl and placed in the control Petri dish. This was repeated for all of the treatment Petri dishes. These last 2 steps were repeated until each Petri dish contained 30 animals. Petri dishes were returned to the incubator until commencement of test, under standard conditions for culturing test animals. Prior to the start of the test, neonates were removed from each Petri dish. Test animals were removed from each Petri dish, 4 at a time, into replicate vials appropriate to each pre-exposure. The first replicate of every treatment was filled before the second, and so forth. The remainder of the feeding test was performed according to protocol. The test was run concurrently with a reproduction bioassay (using SEA as a food additive) (Chapter 4.2.2 *Test 4*).

Test 5: Comparison of final protocol feeding and reproduction bioassays: c) feeding bioassay with algae (only) diet and reproduction bioassay with FFV additive.

One feeding test was performed according to final test protocols (with a diet of algae-only). Copper concentrations used were: 0 (control), 10, 12, 14, 17 and 20 µg/l. One reproduction test was performed using FFV as an additive (i.e. according to reproduction test protocols). Copper concentrations used were 0 (control), 10, 20, 25, 30 and 35 µg/l. Tests were not performed concurrently.

5.2.2 Cadmium

Tests 1 – 3: Comparison of feeding and reproduction bioassays: a) seaweed extract additive.

Three feeding tests were performed using SEA as a food additive. Cadmium concentrations used were: *Test 1*: 0 (control), 1, 5, 10, 50, 100, 200 and 300 µg/l; *Test 2*: 0 (control), 10, 20, 25, 30, 35 and 40 µg/l; and *Test 3*: 0 (control), 10, 12, 14, 17, 20 and 25 µg/l. Only 4 replicates were used for each treatment in *Tests 1* and *3*. *Test 3* was run concurrent with a reproduction bioassay according to standard protocol (using FFV as a food additive).

Tests 4 - 5. Comparison of final protocol feeding and reproduction bioassays: b) feeding bioassay with algae (only) diet and reproduction bioassay with FFV additive.

Two feeding tests were performed according to final test protocols. Cadmium concentrations used were: *Test 4*: 0 (control), 0.5, 1, 1.7, 3, 5.5 and 10 µg/l; and *Test 5*: 0 (control), 0.2, 0.5, 1, 1.7, 3, 5.5 and 10 µg/l. *Test 1* was run concurrently with a reproduction bioassay according to standard protocol.

5.2.3 Uranium

Tests 1 - 2. Feeding bioassay: seaweed extract additive.

Two feeding tests were performed using SEA as a food additive. Uranium concentrations used were: *Test 1*: 0 (control), 10, 20, 30, 50, 75 and 100 µg/l; and *Test 2*: 0 (control), 100, 200 and 300 µg/l.

5.2.4 Mine release water

Test 1. Comparison of feeding and reproduction bioassays: a) gold mine release water.

A feeding test was performed on local gold mine release water (Appendix 19). Treatments used were: 0 (control), 0.1, 0.3, 1, 3.2 and 10 % dilution. The feeding bioassay was run concurrently with a reproduction bioassay performed by staff at the *eriss* Wetland Risk Assessment laboratory.

Test 2. Comparison of feeding and reproduction bioassays: a) uranium mine release water.

A feeding trial was performed on Ranger Uranium Mine release water. Treatments used were: 0 (control), 0.3, 1, 3.2, 10, 32 and 100 % dilution. The feeding bioassay was run concurrently with a reproduction bioassay performed by staff at the ERA Biological Monitoring laboratory.

5.3 Results

5.3.1 Copper

Feeding inhibition was not detected in any of the copper feeding tests (Table 5 and Figure 8a & 9). This was despite high mortality (≥30 %) in higher treatments of 3 of the 5 tests. There was no effect on the feeding test from pre-exposing the test animals i.e. mortality data for this test lay within the range established from non pre-exposed animals.

In the reproduction tests, there was an effect of copper on total offspring number (Table 5 and Figure 8b & 9). In *Test 1*, the effect on brood size was at a higher concentration than the effect on total offspring, and in

Table 5. Effect of copper concentration (µg/l) upon *M. macleayi* reproduction and feeding: point estimates (95 % CI) for various biological endpoints, and descriptive mortality results.

	Test 1 (SEA)	**Test 2 (SEA)**	**Test 3 (SEA)**	**Test 4 (pre-exposure)**	**Test 5 (final protocols)**
EC5 (Total offspring)	**4.6** (3.1-15)	**22** (5.3-29)	**2.7** (1.2-22)	**3.4** (1.7-25)	**2.6** (1.7–7.9)
EC20 (Total offspring)	**15** (7.9-20)	**29** (na)*	**21** (3.9-23)	**24** (na)	**9.5** (5.7-17)
LC50 (72 h) (Reproduction test)	**31** (19-100)	**na**	**na**	**na**	**26** (23-31)
EC5 (Feeding rate)	**15** (na)	**14** (na)	**5.6** (na)	**>31**	**13** (na)
EC20 (Feeding rate)	**>20**	**>29**	**>33**	**>31**	**>15**
Mortality (Feeding test) (control mortality)	55 % at 30 µg/l; 100 % at 40 µg/l (10 %)	0 % at 29 µg/l (0 %)	15 % at 28 µg/l; 30 % at 33 µg/l (5 %)	5 % at 31 µg/l (0 %)	35 % at 18 µg/l; 100 % at 22 µg/l (0 %)
LC50 range (20 h) (Feeding test)	**20-30**	**>29**	**>33**	**>31**	**18–21**

* Not available

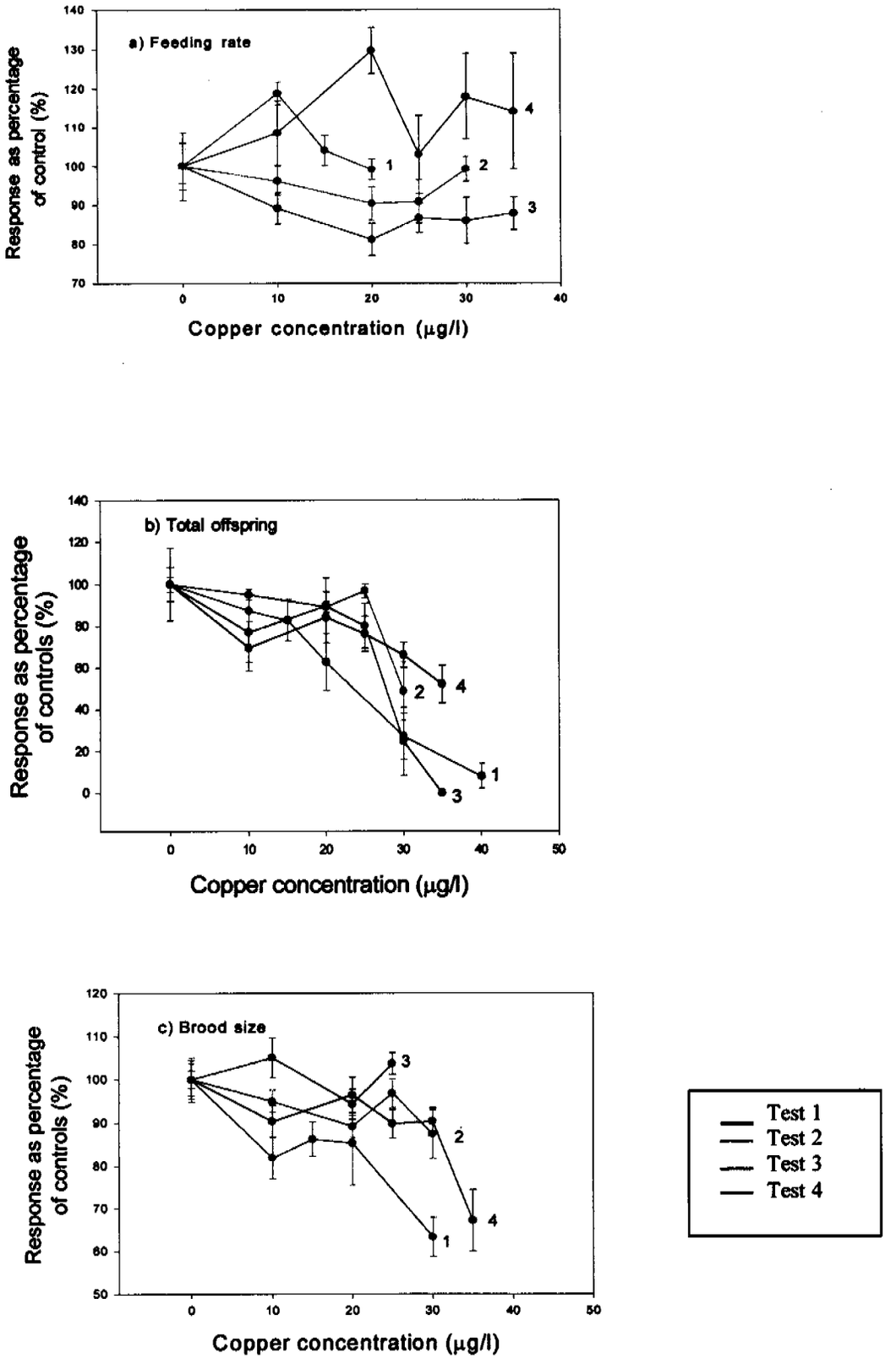

Figure 8. Copper toxicity response (using SEA diet) as percentage of controls (mean (SE)): a) effect on Feeding rate; b) effect on Total offspring; and c) effect on Brood size.

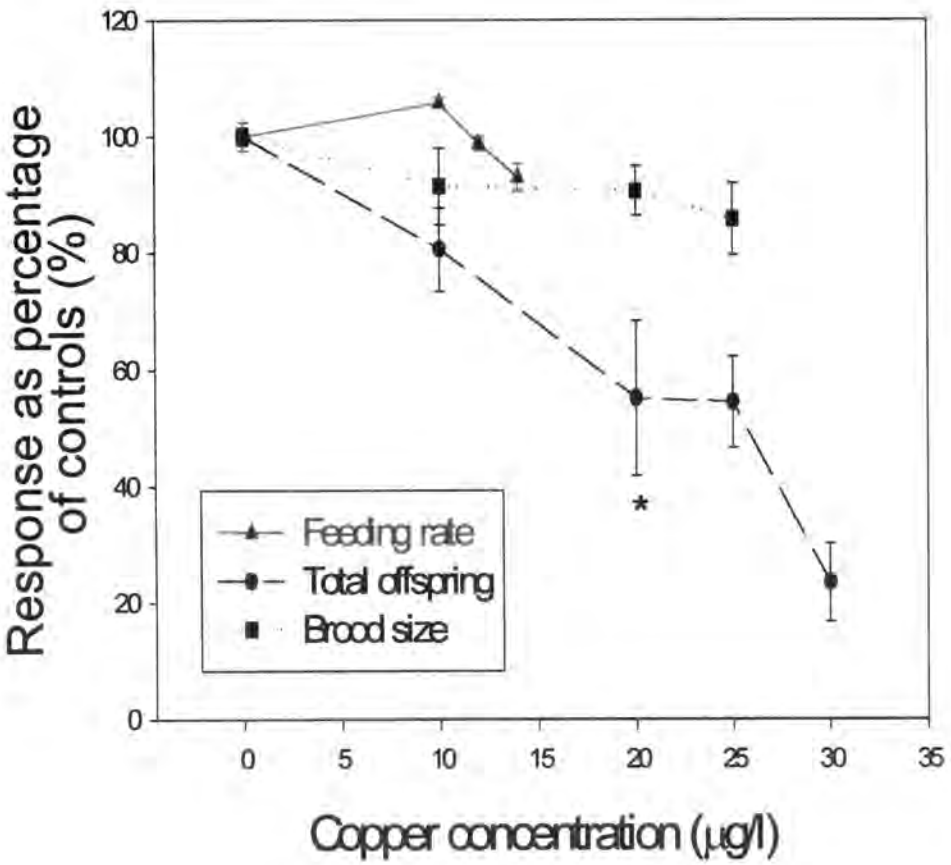

Figure 9. Copper toxicity response (final test protocols) as percentage of controls (mean (SE)): effect on Feeding rate, Total offspring and Brood size. Asterisk (*) indicates lowest concentration that was significantly different from controls (LOEC).

Tests 3 and *5*, no effect was detected on brood size for any of the treatments (Figures 8b & c). In 2 copper reproduction tests performed on 2 different clones of *M. macleayi*, there was no effect of copper on brood size for any treatment, despite an effect being detected on total offspring (Appendix 17).

Where feeding and reproduction tests were run concurrently, and with identical diets (i.e. SEA), total offspring number provided the most sensitive indicator of toxicity. LC50s (20 h mortality) could not be determined from the feeding tests, however, the range was within ×2 of EC20 (total offspring) concentration.

Where feeding and reproduction tests were performed according to protocols, the most sensitive indicator of toxicity was total offspring (Table 5 and Figure 9). Mortality data from both the feeding and reproduction tests were of comparable sensitivity. The LC50 (72 h) and EC50 (total offspring) were not significantly different.

There was no stimulatory effect (i.e. treatment mean higher than control mean) of copper on parameters of total offspring or brood size. In the feeding test, there was a significant stimulatory effect on the lowest treatment (i.e. 10 µg/l) in 2 of the tests.

5.3.2 Cadmium

A strong feeding inhibition response was detected in all cadmium feeding tests (Table 6 and Figure 7 & 10). Although the highest cadmium concentrations were up to an order of magnitude higher than concentrations eliciting feeding effects, low mortality prevented performing probit analysis.

In the reproduction test, total offspring was significantly affected by cadmium (Table 6 and Figure 10). Effects on brood size were not significantly different to effects on total offspring number (Figure 10).

Where the feeding and reproduction tests were performed concurrently and according to protocol, feeding inhibition provided more sensitive LOECs than total offspring (Table 6 and Figure 10). However, when

Table 6. Effect of cadmium concentration (µg/l) upon *M macleayi* reproduction and feeding: point estimates (95 % CI); NOECs - LOECs for various biological endpoints; and descriptive mortality results.

	Test 2 (SEA)	**Test 3 (SEA)**	**Test 4 (final protocols)**	**Test 5 (final protocols)**
EC5 (Feeding rate)	**3.8** (0.6-15)	**3.4** (1.6-9.4)	**0.7** (0.1-1.2)	**0.8** (0.0-1.0)
EC20 (Feeding rate)	**13** (3.7-18)	**12** (7.2-13)	**9.0** (na*)	**5.9** (0.0-7.5)
NOEC-LOEC (Feeding test)	**10.-21**	**10.-12**	**0.5-1.1**	**1.2-1.8**
Mortality (Feeding test) (control mortality)	35 % in 32 and 36 µg/l, 20 % in 21 and 26 µg/l (0 %)	31 % in 27 µg/l (0 %)	0 % in 11 µg/l (0 %)	0 % in 9.6 µg/l (5 %)
EC5 (Total offspring)		**<9.8**	**1.3** (0.9-1.9)	
EC20 (Total offspring)		**<9.8**	**1.8** (1.4-3.2)	
NOEC-LOEC (Reproduction test)		**<0.02-9.8**	**1.7-3.1**	
LC50 range (72 h) (Reproduction test)		**<9.8** (final protocols)	**>10**	

* Not available

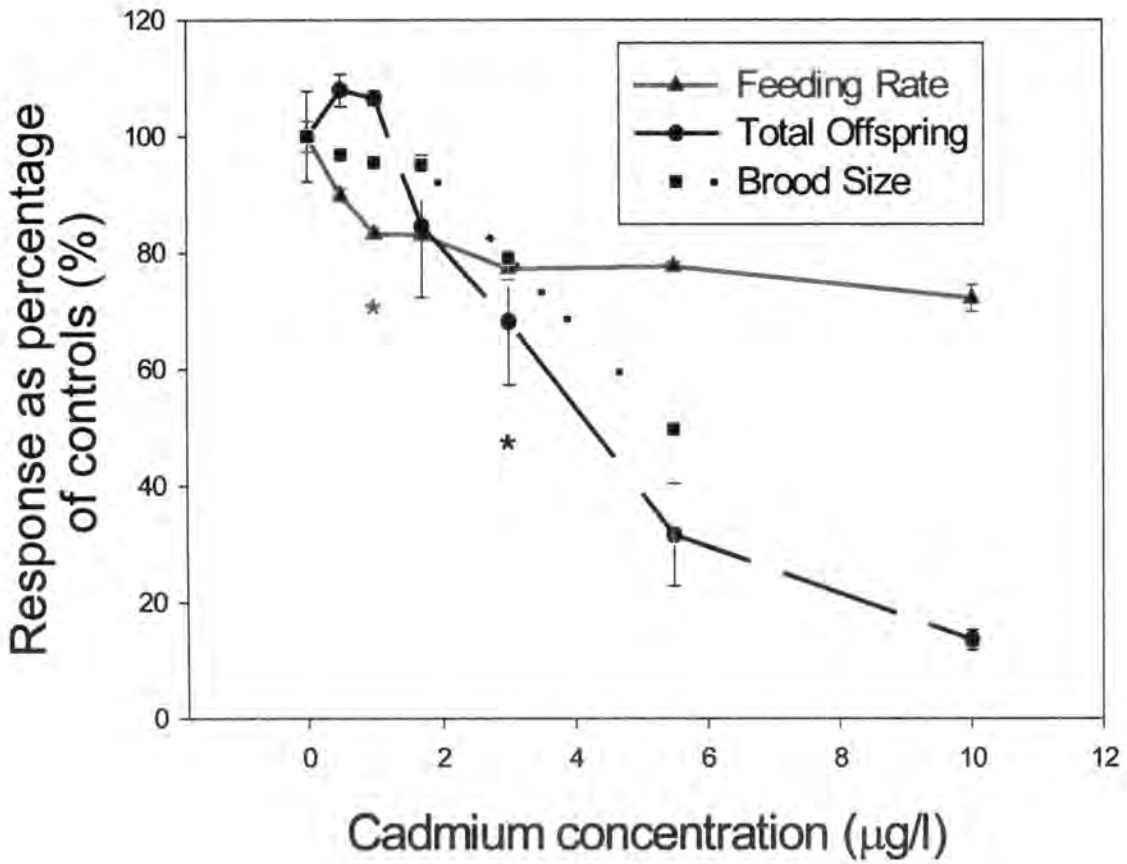

Figure 10. Cadmium toxicity response (final test protocols) as percentage of controls (mean (SE)): effect on Feeding rate, Total offspring and Brood size. Asterisk (*) indicates lowest concentration that was significantly different from controls (LOEC).

point estimates were calculated, total offspring was the more sensitive endpoint (Table 6). This is because a significant effect was detected in the feeding test at <10 % feeding depression (i.e. <EC10).

Cadmium exposure did not have a stimulatory effect on feeding rate. In the reproduction test, there was an increase in total offspring at 0.5 µg/l, however this was not significant.

5.3.3 Uranium

There was no significant effect of uranium exposure on cladoceran feeding rate for either test. There was no mortality in either test.

5.3.4 Mine release water

Gold mine release water. A significant effect of mine water exposure on cladoceran feeding rate was detected (Figure 11a). The feeding test provided a more sensitive NOEC-LOEC (0.3-1 %) than the concurrent reproduction test (1-3.2 %) (*eriss* unpublished data).

Uranium mine release water. A significant effect of mine water exposure on cladoceran feeding rate was detected (Figure 11b). The reproduction test provided a more sensitive NOEC-LOEC (0.3–10 %) (ERA unpublished data) than the concurrent feeding test (32-100 %).

5.4 Discussion

5.4.1 Comparison of results from the present study with toxic responses reported in the literature: Copper

Mortality is considered a sensitive endpoint for measuring copper toxicity, having provided a more sensitive indicator than *r* and offspring number (Winner and Farrell 1976), growth (Dave 1984), observation of enzymatic processes (Janssen and Persoone 1993) and feeding rate (Bitton *et al.* 1996). Even where other parameters have induced effects at lower concentrations, effects upon survival have been within close range (×2) (Flickenger *et al.* 1982; Roux *et al.* 1983). Results from the present study support these findings, with mortality data (from a 20 h feeding test) providing comparable sensitivity to reproduction (over 5 d) as an indicator of toxicity. When tests were run with identical diets, or mortality data examined from the reproduction bioassay, effects upon survival occurred at similar concentrations to

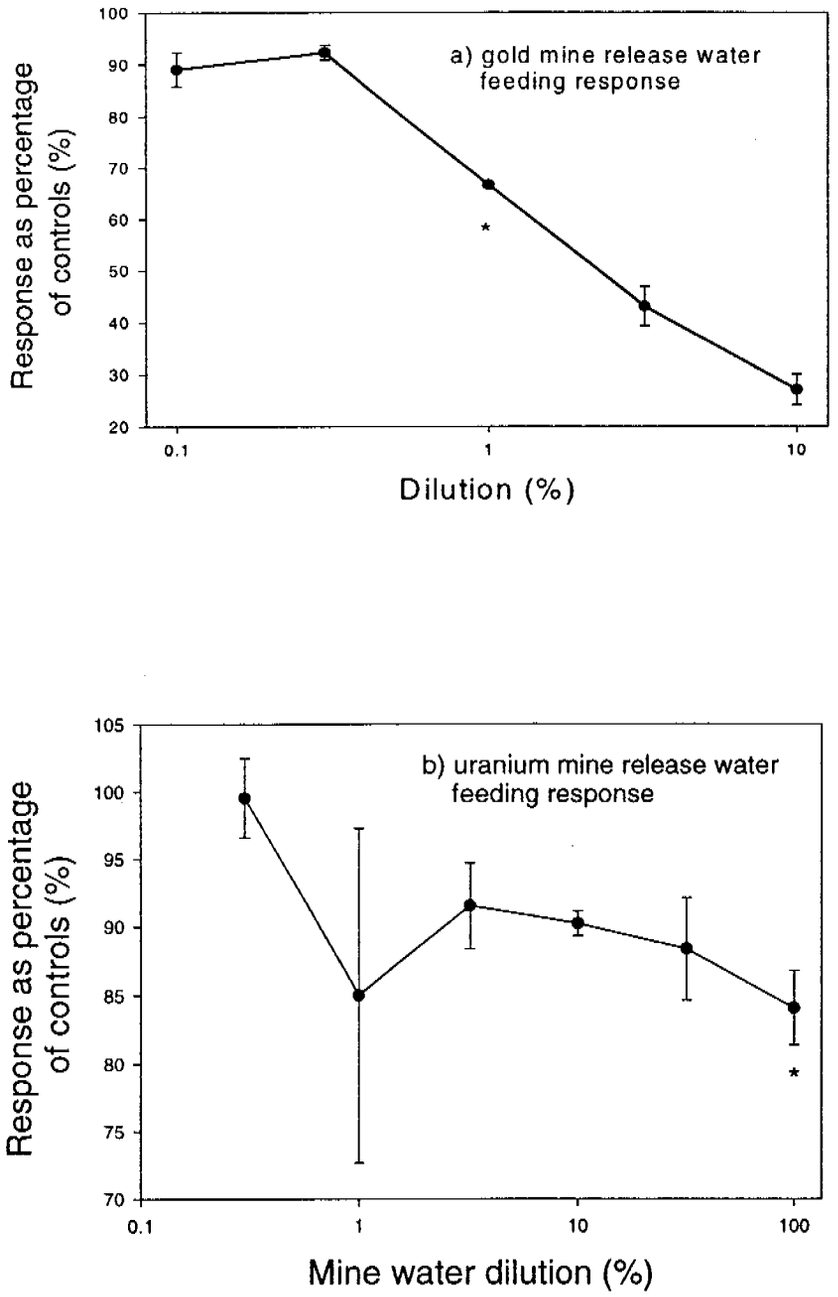

Figure 11. Feeding response as a percentage of controls (mean (SE)) for: a) gold mine release water; and b) uranium mine release water. Asterisk (*) indicates lowest concentration that was significantly different from controls (LOEC).

effects on average fecundity, suggesting that the reduction in offspring numbers could largely be attributed to adult mortality. The results from effects upon brood size corroborated this finding. In 4 of the 7 reproduction tests, there was no effect upon brood size, despite a strong effect upon total offspring. In the other tests, effects to total offspring were stronger than effects to brood size. In addition, it is likely that effects upon brood size have been over-estimated, due to dead neonates often disintegrating before being recorded in the higher treatments. The differences in effects to parameters of total offspring and brood size could also partially be explained by delayed reproduction. Koivisto (1992) studied the effects of copper on 5 species of daphnids. Three of the species suffered no reduction in clutch size or offspring number from exposure. Reduced adult size and delayed reproduction were also reported in some species.

In this study, the feeding test did not provide a sensitive indicator of copper toxicity. However, results from the reproduction test, and interpolation of mortality data show that *M. macleayi* is amongst the 3 most sensitive species (of 14 reported) to copper in a summary list of Australian tropical freshwater species toxicity data (Markich and Camilleri 1997).

Cadmium

The order in endpoint sensitivity was markedly different for cadmium than copper, implying a different toxicant mode of action. Cadmium elicited feeding responses at very low concentrations in comparison to where survival was affected. Similar findings have been reported for *D. magna* cadmium exposure tests where feeding rate (Allen *et al.* 1995; Taylor *et al.* 1998; C. Barata pers. comm.) and enzymatic process (Janssen and Persoone 1993) were affected at an order of magnitude lower than effects to survival.

Feeding inhibition has been reported as a result of cadmium exposure for *D. magna* (Allen *et al.* 1995; Taylor *et al.* 1998; C. Barata pers. comm.). Taylor *et al.* (1998) found that *D. magna* pre-exposed to cadmium contaminated algae had significantly reduced feeding rates 24 h after feeding on clean algae. Animals which were starved in dissolved cadmium, also had slightly reduced feeding rates when moved to clean algae, although feeding rate returned to normal within 24 h. Therefore, although algal-bound cadmium appeared to be the major cause of feeding inhibition, another mode of action occurred when the

animal were exposed to only dissolved cadmium. This suggestion is supported by the findings that the presence of food significantly reduced the sensitivity of *D. magna* to metal toxicity (Chandini 1989; Lanno *et al.* 1989; Taylor *et al.* 1998).

The 2 different methods of statistical analysis used in the present study (point estimates and hypothesis testing) yielded different ranking of endpoint sensitivity for cadmium toxicity. This provides a good example of where statistical models cannot be relied upon alone, to provide complete results, but must be interpreted according to the specific objectives of the testing regime and scrutinised for trends and effects of ecological relevance. In the present study, a 20 % reduction in reproduction occurred at a lower concentration than the same reduction in feeding rate. However, effects to reproduction were not significant at this concentration, whereas feeding rate had been significantly affected at a far lower concentration. One of the main objectives of toxicity testing is to detect effects at low concentrations that have the potential to translate to population level effects. Therefore, a significant 10 % reduction in feeding at very low metal concentrations may be more important than a larger effect to reproduction at higher and possibly more unrealistic toxicant concentrations.

In comparison to other metals, relatively few studies have measured the effects of cadmium on Australian tropical freshwater biota. Lethal endpoint concentrations have been reported for 3 freshwater fish species (Markich and Camilleri 1997). All of these endpoints are between 3-4 orders of magnitude less sensitive than responses determined in this study from the *M. macleayi* feeding and reproduction bioassays.

Uranium

The feeding test did not yield a sensitive result in comparison to reported results from chronic bioassays for *M. macleayi* exposed to uranium in filtered (Magela) Creek water. Rippon (1993) reported LC50s of 200 µg/l (24 h exposure) and 25 µg/l (7 d), illustrating a delayed toxic effect. He acknowledged that it is not usual to use mortality as an endpoint with a chronic test, however sub-lethal effects (reproduction) were not greatly affected by uranium exposure. Delayed mortality as a result of uranium exposure has also been reported for freshwater fish, with kidney damage considered a possible cause (Holdway 1992c).

The diet used in the feeding bioassay may also partially explain the lack of effect detected for uranium. Bywater *et al.* (1991) reported an LC50 (24 h) of 1470 µg/l following the same protocols as Rippon (1993) with the exception of a different food source. In the present study, the sensitivity of test animals to both copper and cadmium was increased when SEA was not used as a food additive, hence, it is possible that a feeding effect may be detected at lower uranium concentrations using final feeding test protocols.

Mine water

The feeding test provided a more sensitive indicator of toxicity than the reproduction test for the gold mine release water, but not the uranium mine release water. The gold mine release water had a high cadmium composition (Appendix 19), which may help explain the strong feeding response detected. The uranium release water had a high uranium component (R. van Dam pers. comm.). Feeding inhibition did not provide a sensitive endpoint for uranium toxicity. Hence, if uranium was a dominant cause of test animal toxicity in the uranium mine release water, it follows that a long-running chronic bioassay would provide a more sensitive indicator of toxicity than a short-exposure feeding test.

In the uranium mine water feeding test, there was a dramatic divergence from the feeding response at 1 % dilution. This manifested in a 15 % feeding depression (not significant) and a coefficient of variation above ×5 the average for other treatments. Linear response modelling has been criticised for its ineffectiveness in accounting for changes in variation around the mean which may carry great ecological significance (Lewontin 1974; Forbes *et al.* 1995). For example, the variation in response of a population may be important in predicting whether a sufficient percentage of the population is likely to survive a particular effect (Holloway *et al.* 1997). In the present case, the response is particular for a very small range, implying that the nature of the chemical species at that dilution (1 %) could potentially induce effects at the population level. This provides an example of the importance of visual assessment of response results, and also chemical speciation modelling, when interpreting toxicity data.

5.4.2 Modes of action

The results from the feeding and reproduction tests, suggest that the mechanics of toxicity differ for copper and cadmium. This corroborates the findings of Roux *et al.* (1993) who investigated the effects of cadmium and copper on survival and reproduction in *D. pulex*. They hypothesised that the metals would induce different toxic responses due to cadmium being a non-essential metal (therefore an α-type toxicity response expected) and copper being an essential metal (therefore a β-type toxicity response expected). They found that cadmium was almost ×4 less toxic than copper in the acute test, whereas it was 3 orders of magnitude more toxic than copper in the reproduction test. Hence, as in the present research, cadmium induced a stronger sub-lethal effect than copper.

Toxicant exposure had an effect on brood size for cadmium, but little (if any) effect for copper. Feeding rate was also affected following cadmium exposure, though not for copper. These findings support models which show that reduction in feeding will directly result in a reduction in reproduction (Kooijman 1986; McCauley *et al.* 1990; Bradley *et al.* 1991). However, it is also possible that the primary effect of a toxicant on brood sizes is due to direct lethal effects on the developing eggs in the brood chamber, and not as a result of sub-lethal effects upon the parent (Baird *et al.* 1991a). It has been demonstrated that food limitation causes a reduction in brood sizes in *D. magna* (Bradley *et al.* 1991). Hence, brood size reductions may be a result of both sub-lethal (to parent) and lethal (to eggs) effects.

C. Barata (pers. comm.) exposed *D. magna* to 3, 4 dichloroaniline (DCA), cadmium and fluoroanthene over 1 and 2 instar exposures and measured egg mortality, feeding rate, offspring production, brood mass and body mass. For DCA, egg mortality was the most sensitive endpoint, but for both cadmium and fluoroanthene, feeding rate was a more sensitive indicator of toxicity than egg mortality, and was at least as sensitive as all other parameters. These results demonstrate the effectiveness of assessing a series of responses via a battery of tests in order to detect differing modes of actions.

5.4.3 Grouping toxicants

If dominant modes of action can be established for different organisms and toxicants, it may be possible to group toxicants according to their physiological and ecological effects. This technique may help not only with ecotoxicological testing regimes and guidelines, but could also potentially be developed as a diagnostic tool. For example, feeding rate and reproductive effects produced different rankings of endpoint sensitivity for the 2 mine release water feeding tests. This suggests that the major mode of action differed between the 2 waste waters. It is therefore possible that with a greater understanding of toxicant effects, such responses, among a battery of tests, may provide insight into the chemical (or chemical group) primarily responsible for the impact.

5.4.4 Energy allocation

One of the advantages of studying feeding is that it provides information regarding the total energy ingested by an animal under various conditions. When used in addition to data on other life history characteristics, this information will aid in understanding the energetics of animals under toxic stress. It has been reported that cladocerans may alter their energy allocation and reproductive strategies when exposed to a toxicant (Kooijman 1986; McCauley *et al.* 1990; Bradley *et al.* 1991; Glazier and Calow 1992). The results from the copper and cadmium feeding and reproduction tests support the hypothesis of Bradley *et al.* (1991), that under food limitation, energy will be directed away from reproduction. This suggests a possible relationship between feeding and reproductive responses, reiterating the need for further investigation of causes and effects of feeding responses.

In 2 of the copper feeding tests, increased feeding was detected at the lowest treatment concentration (lower than any effects were detected for reproduction). At low concentrations, stimulatory effects of copper have been reported for other cladocerans for parameters of brood size (Winner and Farrell 1976) and population growth rate (Koivisto 1992; Roux *et al* 1993). Elevated filtering rates have also been reported for cladocerans exposed to selenium at concentrations where no significant effect was detected on reproduction (Reading and Buikema Jr. 1980). Such effects are generally interpreted as results of hormesis. However, similar responses have also been reported for non-essential metals such as cadmium (Bodar *et*

*al.*1988b; Roux *et al.* 1993). This intimates that an increase in feeding may not be a stimulatory effect but rather a result of increased energy being required to ameliorate protein or other damage. In the present study, the increased feeding did not translate to increased reproduction. This suggests that the increased consumption was required for increased maintenance costs.

5.4.5 Bottom-up analysis - implications for population dynamics and community structure

The strong feeding inhibition response detected for cadmium and multitoxicant (mine release) water has implications both for laboratory bioassays, and for the prediction of environmental effects. It is generally understood that models of population dynamics and community structure calculated from information on individual organisms cannot confidently quantify the influence of indirect effects (Borgmann *et al.* 1989; Gurney *et al.* 1996). It is nevertheless important to assess which indirect effects are likely to occur, and on what scale.

Kooijman *et al.* (1989) stressed the importance of establishing how toxic stress affected feeding in order to model population level effects. They suggested that if the major consequence of toxicant exposure is reduced food intake, toxic effects are not likely to be observed at the population level where food is naturally limited.

Alternatively, it has been suggested by several authors that a reduction in feeding by grazers is likely to have effects not only at the individual and population level, but also indirectly upon the community due to increased algal growth (Jones *et al.* 1991; Glazier and Calow 1992; Bitton *et al.* 1996). Mesocosm test research has supported this hypothesis, demonstrating phytoplankton blooms, as well as increases in other populations that have been released from competition (Borgmann *et al* 1989; Van den Brink *et al.* 1996; Jak *et al.* 1996).

Chapter Six: Summary

6.1 Overview

1. Feeding bioassay protocols have been developed for *M. macleayi* exposed to copper and cadmium.
2. Copper exposure had no effect on the feeding rate of *M. macleayi*. The most sensitive indicators of copper toxicity were total offspring (EC5: 2.6 µg/l; EC20: 9.5 µg/l; NOEC- LOEC: 9.2- 19 µg/l) and mortality (LC50 (20 h): between 18-22 µg/l). The reduction in total offspring can largely be attributed to test animal mortality and delayed reproduction.
3. Cadmium exposure had an effect on the feeding rate of *M. macleayi*. The most sensitive indicators of toxicity were feeding rate (mean EC5: 0.7 µg/l; mean EC20: 7.4 µg/l; mean NOEC-LOEC: 0.9-1.4 µg/l) and total offspring (EC5: 1.3 µg/l; EC20: 1.8 µg/l; NOEC-LOEC: 1.7-3.1 µg/l). Mortality was not a sensitive indicator. The reduction in total offspring can largely be attributed to a reduction in brood sizes.
4. Uranium exposure had no effect on the feeding rate of *M. macleayi* at concentrations ≤290 µg/l when a diet of *Chlorella* sp. and seaweed extract additive was provided. Hence, feeding rate is not a sensitive indicator of uranium toxicity (using this feeding regime) in comparison to reported results using endpoints of mortality and reproduction.
5. The feeding bioassay was a more sensitive indicator of toxicity than the reproduction bioassay following *M. macleayi* exposure to a sample of gold mine release water, but a less sensitive indicator of toxicity following exposure to a sample of uranium mine release water.
6. The diet used in toxicity bioassays has the potential to have a significant effect on results. *M. macleayi* has an increased sensitivity to copper and cadmium when a diet of *Chlorella* sp. (only) is used, in comparison to a diet supplemented with a food additive.

6.2 Future Research

1. Principle aims of this research were to develop a simple and sensitive bioassay. This has been achieved, however, the importance of these objectives necessitates further research directed at refining protocols even further. Examples of modifying factors which could potentially affect test sensitivity are: age of test animal, diet and water type.
2. ASTM was found to be an inadequate culturing medium for test animals. However, further trials with food additives may ameliorate this problem and lead to the development of a successful synthetic water culturing and testing regime. A feeding test using synthetic water would be beneficial for minimising between test variation, and increasing the application of the test.
3. The feeding test did not provide a sensitive indicator of toxicity for uranium. However, the use of SEA was found to decrease the sensitivity of test animals to the metals copper and cadmium. Therefore, it is possible that an uranium-exposure feeding test performed without SEA may have increased sensitivity. The development of sensitive uranium toxicity bioassays is of utmost importance, particularly in the NT, as is a greater understanding of the processes of uranium toxicity to freshwater organisms.
4. The present research further illustrated the significant effects of modifying factors on toxicity bioassays. The developed feeding test provides a simple and rapid procedure that could be used not only for measuring the effects of toxicants, but also the effects of various modifying factors.
5. Previous research has suggested that metal exposure can induce feeding inhibition in cladocerans. This study has provided support for this hypothesis for cadmium (but not copper), and also developed a procedure for testing this theory for a range of toxicants. An increased knowledge of toxicant mode of action will potentially enable the development of more efficient, sensitive, and possibly diagnostic, testing regimes. In addition, it may lead to a greater understanding of the processes and effects of toxicity from the individual, to the community level.

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Appendix 1

Water Collection Dates and Test Water Parameters

Table A1.1. Water collection dates and water parameters.

Water	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH
7/7/98 unfiltered	17	96	6.3
filtered	16	85	6.6
17/9/98 unfiltered	17	93	6.0
filtered	17	98	6.5
15/10/98 unfiltered	18	88	5.6
filtered	18	85	6.5
13/11/98 unfiltered	19	99	6.1
filtered	18	113	6.5
9/12/98 unfiltered	11	91	5.9
filtered	11	88	5.9

Table. A1.2. Test water parameters.

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Chapter 3.3.1		NR*	NR	NR	NR
Chapter 3.3.2 *Test 1*		NR	NR	NR	NR
Chapter 3.3.2 *Test 2*		NR	NR	NR	NR
Chapter 3.3.3 *Test 1*		NR	NR	NR	NR
Chapter 3.3.3 *Test 2*	0 µg/l Cu (ASTM)	153 - 155	99	6.7 - 6.9	34 - 36
	30 µg/l Cu (ASTM)	156 - 158	97 - 99	6.4 - 7.2	36 - 35
	60 µg/l Cu (ASTM)	155	96 - 97	6.6 - 7.8	34 - 35
	Unfiltered creek water	16	98	6.2	NR
	0 µg/l Cu (creek)	23 - 24	97 - 99	7.0 - 8.2	12 - 5.9
	30 µg/l Cu (creek)	23 - 71	98 - 99	- 7.4	8.9 - 4.8
	60 µg/l Cu (creek)	22 - 68	98 - 94	7.1 - 7.3	7.0 - 5.7

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Chapter 3.3.3	ASTM (blank)	148 - 153	95 - 99	9.0 - 8.1	NR
Test 3	ASTM (algae)	160	94 - 96	9.1 - 7.9	
	Unfiltered creek water	16	98	6.2	
	Creek (2.5 µm) (blank)	15	97 - 104	6.5	
	Creek (2.5 µm) (algae)	20	91 - 100	6.6	
	Creek (10 µm) (blank)	15	94 - 108	6.5 - 6.7	
	Creek (10 µm) (algae)	20	94 - 111	6.7 - 6.8	
Chapter 4.2.2	Unfiltered creek water	16	98	6.2	NR
Test 1	0 µg/l Cu	20 - 23	98 - 125	5.1 - 8.0	
	10 µg/l Cu	20 - 22	99 - 125	6.6 - 8.1	
	15 µg/l Cu	20 - 21	95 - 127	6.6 - 8.2	
	20 µg/l Cu	20 - 23	94 - 123	6.6 - 7.8	
	30 µg/l Cu	20 - 24	94 - 126	6.7 - 7.3	
	40 µg/l Cu	20 - 22	93 - 127	6.7 - 7.3	
	50 µg/l Cu	20 - 23	99 - 102	7.1 - 6.8	
Chapter 4.2.2	Unfiltered creek water	18	88	5.6	NR
Test 2	0 µg/l Cu (FFV)	20 - 23	93 - 115	- 7.5	
	0 µg/l Cu	23 - 24	95 - 119	7.6	
	10 µg/l Cu	22 - 25	94 - 120	- 7.5	
	20 µg/l Cu	23 - 24	94 - 119	- 7.4	
	25 µg/l Cu	23 - 24	93 - 119	6.8 - 7.4	
	30 µg/l Cu	23 - 24	93 - 125	6.8 - 7.3	
	35 µg/l Cu	23 - 24	92 - 117	6.9 - 7.4	
Chapter 4.2.2	Unfiltered creek water	17	120	6.6	NR
Test 3	0 µg/l Cu (FFV)	19 - 22	94 - 116	5.8 - 6.9	
	0 µg/l Cu	22 - 24	93 - 120	6.3 - 7.3	
	10 µg/l Cu	22 - 26	100 - 122	6.6 - 7.2	
	20 µg/l Cu	22 - 26	93 - 117	6.8 - 7.1	
	25 µg/l Cu	22 - 28	93 - 118	6.8 - 7.0	
	30 µg/l Cu	21 - 27	93 - 118	6.8 - 7.1	
	35 µg/l Cu	22	105	6.8 - 7.1	
Chapter 4.2.2	Unfiltered creek water	18	96	6.1	NR
Test 4	0 µg/l Cu (FFV)	20 - 23	101 - 118	6.0 - 7.5	
	0 µg/l Cu	23 - 24	98 - 118	6.5 - 7.8	
	10 µg/l Cu	23 - 24	98 - 116	6.7 - 7.9	
	20 µg/l Cu	22 - 24	97 - 117	6.8 - 7.7	
	25 µg/l Cu	23 - 25	99 - 115	6.8 - 7.5	
	30 µg/l Cu	23 - 26	98 - 119	6.8 - 7.6	
	35 µg/l Cu	22 - 24	97 - 116	6.8 - 7.4	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Chapter 4.2.3	Unfiltered creek water	18	102	6.4	NR
Test 1	0 µg/l Cu (ALGAE)	19 - 20	98 - 103	- 6.5	
	5 µg/l Cu (ALGAE)	18 - 19	98 - 106	6.5 - 6.6	
	10 µg/l Cu (ALGAE)	18 - 19	100 - 106	6.5	
	20 µg/l Cu (ALGAE)	18 - 19	98 - 108	6.4 - 6.5	
	40 µg/l Cu (ALGAE)	18 - 19	96 - 107	6.4 - 6.6	
	0 µg/l Cu (SEA)	25	95 - 112	6.6	
	5 µg/l Cu (SEA)	22 - 24	98 - 110	6.7	
	10 µg/l Cu (SEA)	23 - 25	100 - 107	6.6 - 6.7	
	20 µg/l Cu (SEA)	23 - 24	97 - 111	6.6 - 6.7	
	40 µg/l Cu (SEA)	23 - 24	100 - 107	6.6 - 6.7	
Chapter 4.2.3	Unfiltered creek water	17	107	6.5	NR
Test 2	0 µg/l Cd (ALGAE)	18 - 21	99 - 110	6.5 - 6.6	
	5 µg/l Cd (ALGAE)	17 - 21	100 - 108	6.5 - 6.6	
	10 µg/l Cd (ALGAE)	17 - 18	100 - 108	6.5 - 6.6	
	20 µg/l Cd (ALGAE)	17 - 20	99 - 108	6.5 - 6.6	
	0 µg/l Cd (SEA)	23 - 26	98 - 110	6.7	
	5 µg/l Cd (SEA)	23 - 26	98 - 108	6.5 - 6.6	
	10 µg/l Cd (SEA)	23 - 25	100 - 108	6.5 - 6.6	
	20 µg/l Cd (SEA)	23 - 25	100 - 104	6.5 - 6.6	
	0 µg/l Cd (FFV)	21 - 22	96 - 111	6.6	
	5 µg/l Cd (FFV)	21	100 - 108	6.6	
	10 µg/l Cd (FFV)	21 - 26	100 - 107	6.6	
	20 µg/l Cd (FFV)	21 - 22	100 - 106	6.7 - 6.8	
Chapter 5.2.1	Unfiltered creek water	17	81	5.9	NR
Test 1	0 µg/l Cu	20	95 - 100	6.0 - 6.4	
	10 µg/l Cu	20 - 21	96 - 102	6.4 - 6.7	
	15 µg/l Cu	20	96 - 104	6.6 - 6.7	
	20 µg/l Cu	20	94 - 105	6.7	
	30 µg/l Cu	20	92 - 104	6.7	
	40 µg/l Cu	20	92 - 104	6.7 - 6.8	
	50 µg/l Cu	20 - 21	91 - 104	6.7 - 6.8	
Chapter 5.2.1	Unfiltered creek water	18	NR	6.5	NR
Test 2	0 µg/l Cu	24 - 26	97 - 99	- 6.7	
	10 µg/l Cu	24 - 25	95 - 101	6.8 - 6.9	
	20 µg/l Cu	23 - 25	97 - 102	6.9	
	25 µg/l Cu	24	98 - 103	6.9 - 7.0	
	30 µg/l Cu	24	98 - 102	7.0 - 7.1	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Chapter 5.2.1	Unfiltered creek water	18	NR	6.5	NR
Test 3	0 µg/l Cu	23 - 24	95 - 101	5.4 - 6.1	
	10 µg/l Cu	22 - 23	92 - 100	6.5	
	20 µg/l Cu	22	92 - 100	6.7 - 6.8	
	25 µg/l Cu	22 - 23	94 - 99	6.9	
	30 µg/l Cu	22 - 23	95 - 100	6.9	
	35 µg/l Cu	22 - 23	93 - 100	6.9 - 7.0	
Chapter 5.2.1	Unfiltered creek water	19	108	6.8	NR
Test 4	0 µg/l Cu	24 - 25	97 - 112	6.2 - 7.0	
	10 µg/l Cu	23 - 24	94 - 112	6.7 - 7.1	
	20 µg/l Cu	23 - 25	96 - 112	6.8 - 7.0	
	25 µg/l Cu	24 - 25	96 - 110	6.9 - 7.0	
	30 µg/l Cu	23 - 24	95 - 110	6.9 - 7.0	
	35 µg/l Cu	23 - 24	97 - 109	7.0	
Chapter 5.2.1	Unfiltered creek water	14	97	6.4	NR
Test 5	0 µg/l Cu	14 - 16	92 - 100	6.2 - 6.5	
(Feeding test)	10 µg/l Cu	14 - 15	93 - 100	6.4 - 6.5	
	12 µg/l Cu	14 - 15	94 - 100	6.5	
	14 µg/l Cu	14	92 - 102	6.4 - 6.5	
	17 µg/l Cu	14	92 - 109	6.4 - 6.5	
	20 µg/l Cu	14	89 - 99	6.4 - 6.5	
Chapter 5.2.1	Unfiltered creek water	18	111	5.6	NR
Test 5	0 µg/l Cu	20 - 26	101 - 113	6.0 - 7.3	
(Reproduction	10 µg/l Cu	20 - 24	94 - 117	6.4 - 7.3	
test)	20 µg/l Cu	20 - 25	93 - 117	6.0 - 7.3	
	25 µg/l Cu	21 - 24	94 - 115	6.3 - 7.4	
	30 µg/l Cu	20 - 24	93 - 117	6.5 - 7.4	
	35 µg/l Cu	21 - 24	94 - 114	6.5 - 7.5	
Chapter 5.2.2	Unfiltered creek water	18	110	6.4	NR
Test 1	0 µg/l Cd	23 - 24	93 - 94	6.2 - 6.5	
	1 µg/l Cd	23	95 - 98	6.6 - 6.7	
	5 µg/l Cd	23	94 - 102	6.8	
	10 µg/l Cd	23 - 24	97 - 102	6.8 - 6.9	
	50 µg/l Cd	23 - 24	91 - 96	6.8 - 6.9	
	100 µg/l Cd	23	92 - 97	6.9 - 7.0	
	200 µg/l Cd	23	91 - 95	6.8 - 7.0	
	300 µg/l Cd	23	93 - 99	6.8 - 7.0	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Chapter 5.2.2 *Test 2*	Unfiltered creek water	18	101	6.2	NR
	0 µg/l Cd	23	95 - 104	6.7 - 7.0	
	10 µg/l Cd	22 - 23	93 - 105	6.8 - 7.0	
	20 µg/l Cd	23	91 - 105	6.8 - 7.0	
	25 µg/l Cd	23	92 - 103	6.8 - 7.0	
	30 µg/l Cd	23	92 - 105	6.8 - 7.0	
	35 µg/l Cd	23	91 - 104	6.9 - 7.0	
	40 µg/l Cd	23	91 - 102	6.9 - 7.0	
Chapter 5.2.2 *Test 3* (Feeding test)	Unfiltered creek water	18	106	6.8	NR
	0 µg/l Cd	23 - 24	97 - 100	6.9 - 7.2	
	10 µg/l Cd	24	98 - 102	6.9 - 7.1	
	12 µg/l Cd	23	97 - 102	6.9 - 7.1	
	14 µg/l Cd	24	98 - 102	6.9 - 7.0	
	17 µg/l Cd	23 - 24	98 - 101	6.9 - 7.0	
	20 µg/l Cd	23 - 24	98 - 101	6.9 - 7.0	
	25 µg/l Cd	23 - 24	97 - 101	6.9 - 7.0	
Chapter 5.2.2 *Test 3* (Reproduction test)	Unfiltered creek water	18	99	6.3	NR
	0 µg/l Cd	20 - 26	91 - 113	5.7 - 7.3	
	10 µg/l Cd	20 - 21	103 - 122	6.6 - 8.0	
	12 µg/l Cd	20 - 21	105 - 118	6.7 - 8.2	
	14 µg/l Cd	20 - 22	106 - 110	6.8 - 8.3	
	17 µg/l Cd	20 - 21	104 - 110	6.7 - 8.3	
	20 µg/l Cd	20 - 21	105 - 109	6.8 - 8.2	
	25 µg/l Cd	20 - 21	102 - 113	6.8 - 8.4	
Chapter 5.2.2. *Test 4* (Feeding test)	Unfiltered creek water	18	103	5.9	NR
	0 µg/l Cd	18 - 19	96 - 100	6.2 - 6.3	
	0.5 µg/l Cd	18 - 19	99 - 104	6.4 - 6.5	
	1 µg/l Cd	18	97 - 103	6.4 - 6.5	
	1.7 µg/l Cd	18 - 19	98 - 103	6.4 - 6.6	
	3 µg/l Cd	18 - 19	98 - 104	6.4 - 6.5	
	5.5 µg/l Cd	18	99 - 105	6.4 - 6.5	
	10 µg/l Cd	18 - 19	98 - 102	6.4 - 6.5	
Chapter 5.2.2 *Test 4* (Reproduction test)	Unfiltered creek water	18	111	5.6	NR
	0 µg/l Cd	21 - 23	94 - 111	6.2 - 6.9	
	0.5 µg/l Cd	21 - 22	94 - 120	6.4 - 7.0	
	1 µg/l Cd	20 - 21	93 - 120	6.4 - 7.0	
	1.7 µg/l Cd	20 - 22	94 - 120	6.5 - 7.1	
	3 µg/l Cd	20 - 22	94 - 117	6.5 - 7.1	
	5.5 µg/l Cd	20 - 22	94 - 128	6.3 - 7.1	
	10 µg/l Cd	20 - 21	97 - 128	6.6 - 7.3	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Chapter 5.2.2	Unfiltered creek water	19	114	6.3	NR
Test 5	0 µg/l Cd	18 - 19	95 - 99	6.0 - 6.5	
	0.2 µg/l Cd	17 - 18	98 - 103	6.4 - 6.6	
	0.5 µg/l Cd	18	98	6.4 - 6.5	
	1 µg/l Cd	18 - 23	97 - 102	6.5	
	1.7 µg/l Cd	17 - 18	98 - 105	6.5	
	3 µg/l Cd	17	95 - 101	6.5	
	5.5 µg/l Cd	17	97 - 105	6.5	
	10 µg/l Cd	17 - 18	99 - 103	6.5	
Chapter 5.2.3	Unfiltered creek water	18	103	6.2	NR
Test 1	0 µg/l U	23	94 - 100	6.7 - 7.0	
	10 µg/l U	23	98 - 99	6.8 - 7.0	
	20 µg/l U	23	98 - 99	6.9 - 7.0	
	30 µg/l U	23	96 - 97	6.9 - 7.0	
	50 µg/l U	23	98 - 99	6.9 - 7.0	
	75 µg/l U	23	98 - 100	6.9 - 7.0	
	100 µg/l U	23	98 - 101	7.0	
Chapter 5.2.3	Unfiltered creek water	18	105	6.3	NR
Test 2	0 µg/l U	24	99 - 101	6.5 - 6.9	
	100 µg/l U	23	98 - 103	6.6 - 6.8	
	200 µg/l U	23	98 - 103	6.6 - 6.7	
	300 µg/l U	23	98 - 103	6.7 - 6.8	
Chapter 5.2.4	Unfiltered creek water	18	111	5.8	NR
Test 1	0 %	17 - 19	98 - 105	6.3 - 6.6	
	0.1%	19 - 22	98 - 104	6.3 - 6.6	
	0.3 %	23 - 26	96 - 107	6.2 - 6.5	
	1 %	36	98 - 108	5.5 - 5.8	
	%	83 - 84	96 - 107	4.4 - 4.5	
	10 %	218 - 223	98 - 105	3.9 - 4.0	
Chapter 5.2.4	Unfiltered creek water	13	100	5.4	NR
Test 2	0 %	15 - 17	95 - 103	6.0	
	0.3 %	16 - 25	95 - 105	6.0 - 6.3	
	1 %	29 - 33	94 - 108	6.3 - 6.5	
	3.2 %	56 - 58	91 - 110	6.5 - 6.6	
	10 %	141 - 142	96 - 107	6.8 - 7.1	
	32 %	392 - 393	95 - 106	7.2 - 7.3	
	100 %	1001 - 1006	97 - 107	7.8 - 8.0	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Appendix 14 *Tests 1-2*		NR	NR	NR	NR
Appendix 14 *Test 3*	0 µg/l Cu			7.3	NR
	1 µg/l Cu			7.4	
	2 µg/l Cu			7.5	
	4 µg/l Cu			7.6	
	7.5 µg/l Cu			7.6	
	15 µg/l Cu			7.5	
	30 µg/l Cu			7.6	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Appendix 14 *Test 4*	0 µg/l Cu	161 - 173	NR	7.2 - 7.7	33 - 34
	10 µg/l Cu	152 - 167		7.2 - 7.5	32 - 35
	15 µg/l Cu	160 - 167		7.1 - 7.5	32 - 34
	20 µg/l Cu	161 - 167		7.3 - 7.5	32 - 35
	30 µg/l Cu	156 - 68		7.2 - 7.7	34 - 36
	40 µg/l Cu	163 - 68		7.2 - 7.7	34 - 36
	60 µg/l Cu	165 - 68		7.3 - 7.7	34 - 35
Appendix 14 *Test 5*	0 µg/l Cu	118 - 119	NR	7.3 - 7.6	NR
	10 µg/l Cu	176 - 177		7.8	
	15 µg/l Cu	178		7.8 - 7.9	
	20 µg/l Cu	177 - 178		7.8 - 8.0	
	30 µg/l Cu	177 - 178		7.8 - 7.9	
	60 µg/l Cu	177		7.8 - 8.0	
	100 µg/l Cu	151		7.8 - 7.9	
Appendix 15 - 16		NR	NR	NR	NR
Appendix 17 (Reproduction test - RP1 clone)	Unfiltered creek water	18	118	6.5	NR
	0 µg/l Cu	20 - 22	96 - 123	6.2 - 7.9	
	10 µg/l Cu	20 - 22	96 - 125	6.5 - 8.0	
	20 µg/l Cu	20 - 22	94 - 122	6.6 - 8.0	
	25 µg/l Cu	21 - 22	96 - 120	6.7 - 7.7	
	30 µg/l Cu	21 - 22	94 - 108	7.1 - 7.5	
	35 µg/l Cu	21	96 - 103	7.1 - 7.5	
	40 µg/l Cu	21 - 22	90 - 102	7.0 - 7.4	

Test	Treatment	Conductivity (µS/cm)	Dissolved Oxygen (%)	pH	Alkalinity (mg $CaCO_3$/l)
Appendix 17 (Reproduction test - BB clone)	Unfiltered creek water	21	103	5.7	NR
	0 µg/l Cu	21 - 23	100 - 118	6.0 - 6.9	
	10 µg/l Cu	21 - 23	97 - 121	6.1 - 7.1	
	20 µg/l Cu	21 - 23	97 - 118	6.2 - 7.0	
	25 µg/l Cu	20 - 23	98 - 121	6.2 - 7.0	
	30 µg/l Cu	21 - 25	99 - 123	6.1 - 7.1	
	35 µg/l Cu	20 - 23	98 - 127	6.3 - 7.2	
Appendix 17 (Feeding test - RP1 clone)	Unfiltered creek water	18	114	6.7	NR
	0 µg/l Cd	19 - 22	95 - 101	6.4 - 6.6	
	0.5 µg/l Cd	18	96 - 99	6.7	
	1 µg/l Cd	18	94 - 101	6.7 - 6.8	
	1.7 µg/l Cd	18	91 - 98	6.7 - 6.8	
	3 µg/l Cd	18 - 20	94 - 99	6.7 - 6.8	
	5.5 µg/l Cd	18 - 19	96 - 99	6.7 - 6.8	
	10 µg/l Cd	19	96 - 102	6.4 - 6.8	
	18 µg/l Cd	18 - 19	94 - 99	6.7 - 6.8	
Appendix 17 (Feeding test - BB clone)	Unfiltered creek water	18	105	6.4	NR
	0 µg/l Cd	18 - 19	97 - 103		
	0.5 µg/l Cd	18	99 - 103		
	1 µg/l Cd	18	98 - 103		
	1.7 µg/l Cd	17 - 18	98 - 103		
	3 µg/l Cd	17 - 18	98 - 103		
	5.5 µg/l Cd	17	99 - 103		
	10 µg/l Cd	18	95 - 100		
	18 µg/l Cd	18	97 - 105		

* Not reported

Appendix 2

Production of Stock Solutions

1. Stock solutions should be prepared from chloride and sulphate salts (e.g. cooper sulphate, cadmium chloride and uranyl sulphate) dissolved in Milli Q water at a concentration of 10 mg/l. Solutions can be refrigerated in plastic screw-capped bottles if it has been determined that solutions do not degrade over time. Otherwise, stock solutions should be made on the morning of the start of the test.
2. On the morning of test commencement, prepare a 1 in 20 solution of stock solutions in filtered test dilution water to produce a 500 µg/l solution.
3. Dispense appropriate volumes of the 500 µg/l solution in filtered test dilution water to produce 500 ml of each required test treatment concentration. These should be mixed in 500 ml screw-capped glass jars.

Appendix 3

Nominal and Actual Metal Concentrations

Table A3.1. Nominal and actual metal concentrations for copper exposure tests.

Test	Time	Nominal Copper Concentration (µg/l)	Actual Copper Concentration (µg/l)
Chapter 4.2.2 *Test 1*	day 0	0	1.5
		10	9.3
		15	14
		20	19
		30	29
		40	36
		50	46
	day 5	0	1.4
		10	8.5
		15	12
		20	19
		30	24
		40	30
Chapter 4.2.2 *Test 2*	day 0	0	0.49
		10	8.7
		20	22
		25	26
		30	29
		35	31
	day 5	0	2.1
		10	8.5
		20	18
		25	20
		30	23
		35	25

Test	Time	Nominal Copper Concentration (µg/l)	Actual Copper Concentration (µg/l)
Chapter 4.2.2 *Test 3*	day 0	0	0.36
		10	7.9
		20	17
		25	21
		30	25
		35	29
	day 6	0	1.5
		10	7.5
		20	15
		25	19
		30	23
		35	27
Chapter 4.2.2 *Test 4*	day 0	0	0.23
		10	9.5
		20	19
		25	23
		30	28
		35	31
	day 5	0	0.51
		10	7.2
		20	15
		25	19
		30	24
		35	27
Chapter 4.2.3 *Test 1*	t_0	0	1.3
		5	5.24
		10	9.5
		20	18
		40	38
Chapter 5.2.1 *Test 1*	t_0	0	0.75
		10	10
		15	15
		20	20
		30	30
		40	40
		50	49
	t_{20}	0	1.3
		10	10
		15	14
		20	19
		30	28
		40	39
		50	48

Test	Time	Nominal Copper Concentration (µg/l)	Actual Copper Concentration (µg/l)
Chapter 5.2.1 *Test 2*	t_0	0	0.74
		10	9.7
		20	20
		25	24
		30	29
Chapter 5.2.1 *Test 3*	t_{20}	0	1.6
		10	10
		20	20
		25	24
		30	28
		35	33
Chapter 5.2.1 *Test 4*	t_0	0	1.4
		10	9.3
		20	19
		25	23
		30	28
		35	31
Chapter 5.2.1 *Test 5* (Feeding test)	t_0	0	2.1
		10	11
		12	13
		14	15
		17	18
		20	21
Chapter 5.2.1 *Test 5* (Reproduction test)	day 0	0	0.36
		10	9.2
		20	19
		25	22
		30	27
		35	33
	day 5	0	0.51
		10	9.1
		20	19
		25	21
		30	27
		35	33
Appendix 14 *Test 1-3*			na[1]

Test	Time	Nominal Copper Concentration (µg/l)	Actual Copper Concentration (µg/l)
Appendix *Test 4*	t_0	0	na
		10	7.4
		15	11
		20	15
		30	23
		40	30
		60	47
	t_{20}	0	na
		10	8.3
		15	12
		20	14
		30	22
		40	26
		60	43
Appendix 14 *Test 5*	t_0	0	0.28
		10	7.3
		15	12
		20	16
		30	23
		60	45
		100	78
	t_{20}	0	0.80
		10	7.0
		15	12
		20	14
		30	21
		60	42
		100	66
Appendix 17 (RP1 clone)	day 0	0	1.2
		10	9.2
		20	18
		25	23
		30	27
		35	36
	day 6	0	1.9
		10	8.8
		20	17
		25	22
		30	26
		35	29

Test	Time	Nominal Copper Concentration (µg/l)	Actual Copper Concentration (µg/l)
Appendix 17 (BB clone)	day 0	0	0.65
		10	9.7
		20	19
		25	23
		30	29
		35	33
		0	1.1
		10	10
		20	19
		25	23
		30	28
		35	32

Table A3.2. Nominal and actual metal concentrations for cadmium exposure tests.

Test	**Time**	**Nominal Cadmium Concentration (µg/l)**	**Actual Cadmium Concentration (µg/l)**
Chapter 4.2.3	t_0	0	0.38
Test 2		5	5.2
		10	9.9
		20	21
Chapter 5.2.2	t_0	0	0.07
Test 1		1	1.1
		5	5.1
		10	10
		50	52
		100	110
		200	210
		300	310
Chapter 5.2.2	t_0	0	0.07
Test 2		10	10
		20	21
		25	26
		30	32
		35	36
		40	42
Chapter 5.2.2	t_0	0	0.05
Test 3		10	10
(Feeding test)		12	12
		14	15
		17	18
		20	21
		25	27
Chapter 5.2.2	day 0	0	<0.02
Test 3		10	9.8
(Reproduction test)		12	12
		14	14
		17	14
		20	20
		25	25
	day 6	0	<0.02
		10	10
		12	12
		14	14
		17	17
		20	20
		25	26

Test	Time	Nominal Cadmium Concentration (µg/l)	Actual Cadmium Concentration (µg/l)
Chapter 5.2.2	t_0	0	<0.02
Test 4		0.5	0.52
(Feeding test)		1	1.1
		1.7	1.8
		2	3.2
		5.5	5.9
		10	11
Chapter 5.2.2	day 0	0	0.08
Test 4		0.5	0.87
(Reproduction test)		1	1.2
		1.7	1.7
		3	3.1
		5.5	5.4
		10	10
	day 5	0	0.10
		0.5	0.94
		1	1.1
		1.7	1.8
		3	3.1
		5.5	5.4
		10	10
Chapter 5.2.2	t_0	0	0.09
Test 5		0.2	0.30
		0.5	0.75
		1.0	1.2
		1.7	1.8
		3.0	3.0
		5.5	5.7
		10	9.6
Appendix 17	t_0	0	0.07
(RP1 clone)		0.5	0.56
		1.0	1.1
		1.7	1.7
		3.0	3.0
		5.5	5.7
		10	10
		18	18

Test	Time	Nominal Cadmium Concentration (µg/l)	Actual Cadmium Concentration (µg/l)
Appendix 17 (BB clone)	t_0	0	0.28
		0.5	0.78
		1.0	1.2
		1.7	1.8
		3.0	3.0
		5.5	5.7
		10	10
		18	19

Table A3.3. Nominal and actual metal concentrations for uranium exposure tests.

Test	Time	Nominal Uranium Concentration (µg/l)	Actual Uranium Concentration (µg/l)
Chapter 5.2.3	t_0	0	<0.01
Test 1		10	8.8
		20	19
		30	27
		50	46
		75	72
		100	96
Chapter 5.2.3	t_0	0	0.09
Test 2		100	94
		200	190
		300	290

[1] Not available

Appendix 4

Feeding Test Procedure and Protocols

Test Procedure

1. About 2 h before the expected commencement time of the test (immediately after test animals have had their first brood), prepare test treatments in 500 ml bottles as outlined in Appendix 2.
2. Dispense 50 ml from each treatment into polyethylene nalgene bottles and acidify to 0.13% HNO_3. These samples are for chemical analysis.
3. Add appropriate volume of *Chlorella* sp. concentrate to each treatment to produce a 2×10^5 cells/ml algal density.
4. Shake treatments and dispense 30 ml aliquots of each of the 7 test solutions into 7×45 ml replicate vials. Treatments should be labelled A-G, and replicates 1-7. Also dispense 30 ml aliquots of filtered dilution water into 2×45 ml vials labelled CB1 and CB2. Pour approximately 80 ml of each treatment and dilution water into 100 ml plastic vials for pH, conductivity and dissolved oxygen measurement.
5. Isolate at least 140 adult fleas from culture bowl into a Petri dish.
6. When replicate solutions have reached 27° C, use an eye-dropper to move 4 fleas from the Petri dish to the first control replicate. Repeat for the first vial of the remaining 6 treatments, working up from lowest to highest concentration.
7. Obtain a clean eye-dropper and repeat step 6 for the next 4 vials, until 5 vials for each of the 7 treatments contains 4 test animals.
8. Cover each vial with the appropriately labelled lid (A-G, 1-5 for treatment replicates; A-G, 6-7 for treatment 'blanks'; CB1-2 for coulter counter test blanks / test dilution water), and place on the perspex trays, in random order. Place the trays in the incubator in random order. Completion of this stage constitutes the start of the test time (t_0).

9. Shake control treatment bottle and take set of counts using Coulter counter protocols (Appendix 6).
10. Repeat step 9 for each treatment (from lowest to highest concentration) and for the test dilution water. Record counts as 't_0'.
11. Measure pH, conductivity and dissolved oxygen for each treatment and for test dilution water and record as t_0. Rinse vials with Milli Q water.
12. After 20 h from test commencement, remove perspex trays from incubator.
13. Remove test animals from the first control replicate using a Pasteur pipette, recording the number of alive and dead adults, as well as presence of neonates. If less than 3 adults are alive, the replicate should be considered invalid for algal cell counts.
14. Follow this procedure for the first vials for the rest of the treatments, and then for the second to fifth vials for each treatment, obtaining a clean pipette prior to each control replicate. All animals should have been removed and status (alive/dead, and neonate presence/absence, recorded).
15. Remove the cap of the first blank control replicate (i.e. A6) and replace it with a clean and intact cap (i.e. no hole drilled through lid). Place a clean nozzle on the pipette. Shake the sample 4 times then take a set of cell counts using the procedure explained in step 9. Rinse the cap with Milli Q water.
16. Repeat for each of the first blanks for the other treatments, starting from the lowest through to the highest concentrations. Record counts as 't_{20}'
17. If the mean count for a replicate is more than 15 % different than the t_0 mean count for that treatment, repeat the counts with a second sample from the outlying replicate. If still outside of the 15 % range, then a set of counts should be taken from the second blank (i.e. labelled 7).
18. Repeat steps 15-17 for the first of the dilution test water vials (i.e. CB1).
19. Follow the procedure of steps 15-16 for the first control vial (i.e. A1), and then for the first replicate of each treatment.
20. Repeat for the second through to the fifth vials for each treatment, so that all vials have been counted.

21. Pour remainder of vials for each treatment (including the dilution test water vials) into the 100 ml vial used for measuring water parameters for that treatment on the previous day. Measure pH, conductivity and dissolved oxygen and record as t_{20}.

Recording of test conditions

pH, conductivity and dissolved oxygen concentration of each test solution should be measured and recorded at the beginning (t_0) and end (t_{20}) of the test.

Acceptability of test data

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

1. the recorded temperature in the incubator remains within 27 ± 1 °C;
2. all control samples are valid (i.e. at least 3 adult animals alive in each replicate);
3. any change in cell counts between t_0 and t_{20} blanks is consistent for all treatments (i.e. within 10 %) e.g. all blanks increased 10 %.

The data set of each group is considered acceptable if:

1. at least 3 of the replicates for a treatment are valid (i.e. at least 3 adults from at least 3 replicates are alive at the end of the test);
2. the t_{20} pH is within ± 0.5 units of the t_0 pH;
3. the dissolved oxygen concentration is >70 % of the air saturation value throughout the test at a temperature of 27 °C;

4. the t_{20} conductivity for each test solution is within ±10 % of the values for t_0 conductivity.
5. If the control is not acceptable according to the above validity criteria then statistical testing should not proceed. Further, statistical testing should not proceed if fewer than 4 groups (including control) remain.

Analysis of test data

1. *Hypothesis testing.* If data conforms to assumptions required for ANOVA analysis (i.e. assumption of homogeneity of variance; assumption of normal distribution; assumption of independent replication) establish maximum NOEC and LOEC using 1-way ANOVA with *post-hoc* pairwise comparison (e.g. Dunnet's Test) (Factor: Toxicant concentration / dilution; Response: Feeding rate). If data does not conform to ANOVA assumptions, then use a non-parametric test (e.g. Steel's Many-One Rank Test).
2. *Point estimates.* Calculate EC5 and EC20 (with 95 % CI) using regression analysis.

Reporting of results

The test report should include the following information:

1. Source of the test water and site, date and time of collection, where applicable. Concentration and ingredients for stock toxicant solution, where applicable
2. Source of the dilution water, and site, date, and time of collection.
3. Method of collection, transport and storage of water
4. Date and time of start of test.
5. Concentrations / dilutions tested
6. Tabulated data showing t_0 and t_{20} pH, dissolved oxygen concentration, and conductivity for each treatment and test dilution water.
7. Tabulated data showing survival and presence of neonates (present / absent) for each replicate
8. Tabulated data showing 4 'narrow range' algal cell counts for 1 'blank' at each test treatment and test dilution water at t_0 and t_{20}, and for each valid replicate at t_{20}.
9. Definition of the NOEC and LOEC for algal feeding rate.
10. EC5 and EC20 (95 % CI).
11. Any deviation from the method as described above.

Appendix 5

Production of Test Animals (Feeding Bioassay)

1. Parents of test animals should be raised according to *M. macleayi* reproduction test protocols (Hyne *et al.* 1996).
2. Test animals should be second brood progeny, released within 6 h of each other. Approximately 200 neonates should be transferred to a covered and aerated bowl containing approximately 1.5 l of test dilution water (filtered at 10 µm), *Chlorella* sp.(2×10^5 cells/ml) and FFV (1 µml/ml).
3. Every 24 h, transfer test animals, using an eye-dropper, to a bowl of fresh culturing solution (step 2).
4. Animals are ready for the start of the test immediately following release of their first brood (day 3 or 4).

Appendix 6

Protocol for taking Coulter Counter Sample Counts

1. Set the Coulter counter to measure 500 µl samples. Prior to taking replicate cell counts, several counts should be run with isotone solution, until the counts are consistently below 800 (cells/500 µl).
2. Dispense 5ml of test solution into the homogeniser.
3. Add 5ml isotone.
4. Homogenise mixture using 4 twisted plunges of the pestle and pour mixture into a 10ml plastic vial.
5. At the beginning of a test, the algal peak must be isolated within the 'narrow range' window. Measure the 'full range' count of the first control blank. Move the 'narrow range' borders to the lowest (y-axis) point on either side of the algal peak.
6. Repeat steps 1-3 for the first treatment blank and take a 'full range' measurement, checking that the window previously set for the control contains the entire algal peak.
7. Once the window has been adjusted so that it is suitable for both control and treatment counts, return to the control sample and record 4 cell counts on 'narrow range'.
8. Only include counts running within ±5 % of established average time duration.
9. All counts must be within ±5 % of each other counts.
10. Rinse plastic vial, pipette nozzle and homogeniser with Milli Q water between samples.
11. If the is a delay or interruption for more than 3 minutes before completing a prepared sample, then that sample should be re-homogenised.
12. If any mean replicate count is not within ±15 % of all other mean replicate counts, then another sample should be taken and counted from the replicate. This should not be done more than once for a single sample.

Appendix 7

Protocol for Calculating Cell Density and Algal Feeding Rate

1. At t_0, Coulter counts should be measured for 1 replicate and 1 coulter counter blank (dilution water) for each treatment. At t_{20}, 4 replicates, 2 blanks (no animals) and 1 Coulter counter blank should be measured.
2. Each sample should be measured according to protocols for taking Coulter counter counts (Appendix 6).
3. Average the counts for each sample and multiply by the appropriate factor to obtain cell density in units of cells/30 ml (i.e. × 20, for 100 µl Coulter counter sample of 1:1 replicate: isotone solution).
4. Subtract dilution water cell density (calculated from the Coulter counter blank) from all of the replicate density calculations appropriate for t_0 and t_{20} samples.
5. Average t_0 and t_{20} blank counts for each treatment.

Calculating cell density

6. Subtract blank counts (step 4) from algal counts (step 3) for each replicate.
7. Calculate mean, standard error and coefficient of variation for each treatment.

Calculating feeding rate

6. Feeding rate is calculated for each replicate using a simplified version of Gauld's equation (Gauld 1951):

$$F = \frac{V.(C_i - C_f)}{n.t}$$

where: F = feeding rate in cells/animal/h

C_i = initial cell concentration (i.e. product of step 4)

C_f = final cell concentration (i.e. product of step 3)

V = volume in ml (i.e. 30)

n = number of animals (i.e. 4)

t = exposure period in h (i.e. 20)

8. For each treatment, mean feeding rate, standard error and confidence of variation should be determined

Appendix 8

Performing Concurrent Reproduction and Feeding Bioassays

1. An additional 500 ml of each of the reproduction test treatment solutions should be made to be used in the feeding test.
2. On the morning the feeding test is to begin, remove this amount from each of the solutions, and pass each through a 2.5 μm filter. Store in glass jars and warm to room temperature until test commencement.
3. The tests require a minimum of 250 second brood neonates, released within 6 h of each other.
4. Separate approximately 100 into a Petri dish and use immediately for a reproduction test.
5. Culture the remaining neonates according the procedure for production of feeding test animals (Appendix 5).

Appendix 9

Temperature

Temperature has been found to have an effect upon cladoceran reproduction (Lee *et al.* 1986) as well as toxicant uptake in algae (Gipps and Coller 1980). In order to maintain consistency with *M. macleayi* culturing and reproduction bioassay protocols, all tests were run in sealed incubators at 27 ± 1°C, except where otherwise stated (i.e. earlier tests, which were performed without the use of incubators).

Appendix 10

Test Duration

Test duration of was initially set at 24 h, conforming with the *D. magna* feeding test (Allen *et al.*, 1995; Taylor *et al.* 1998; C. Barata pers. comm.). As the generation time of *M. macleayi* is significantly shorter than *D. magna*, it was not considered appropriate to trial longer exposures. This is also to comply with principle aims to develop a simple and rapid test regime. However, the test was later shortened, as 20 h was a more convenient exposure time. Hence, except where stated (i.e. earlier tests), all tests reported used 20 h exposure.

Appendix 11

Age of Animal

Cladoceran neonates are considered more sensitive to toxicants than adult animals (Jones *et al.* 1991). However, neonates were not considered for testing on 3 grounds: 1) difficulty in handling due to their minute size, 2) suspected large number of test animals required to produce detectable feeding depression, and 3) research suggesting that neonates are sustained nutritionally largely from energy stored while in the brood pouch (Cowgill 1984). *M. macleayi* usually release their first brood on day 2 or 3 and release subsequent broods every 24-48 h (R. van Dam pers. comm.). The effects of brood release (both parent and neonate feeding regimes) have not been investigated, therefore in order to perform a 20 - 24 h test whilst least chance of confounding results with brood release, test commencement was chosen at immediately after the release of first brood.

Appendix 12

Seaweed Extract Additive

Feeding Solution

120 µl of Stock solution per 30 ml sample OR 4 ml in 1l

Table A12.1. Composition of seaweed extract concentrate

	Marinure 2252 in dry powder form*	**Stock solution+**
Dry matter	92-95 %	
Organic matter	50-55 %	
Inorganic matter	40-45 %	
N	1.4 %	0.007 %
P	0.05 %	0.00025 %
K	2.5 %	0.0125 %
Ca	1.2 %	0.006 %
Mg	0.8 %	0.004 %
S	3.7 %	0.0185 %
Cl	4.0 %	0.02 %
Al	5.0 mg/l	0.025 mg/l
B	82.0 mg/l	0.41 mg/l
Co	1.6 mg/l	0.008 mg/l
Cu	5.0 mg/l	0.025 mg/l
Fe	3000 mg/l	15 mg/l
I	1800 mg/l	9 mg/l
Mn	12 mg/l	0.06 mg/l
Ni	5 mg/l	0.025 mg/l
V	0.7 mg/l	0.0035 mg/l
Zn	100 mg/l	0.5 mg/l
Cytokinins and other growth promoting hormones	130-260 mg/l	0.65–1.3 mg/l

* data supplied by Pann Brittanica Industries, Ltd, UK

+ 5 ml in 1l Distilled Water

Appendix 13

Three-way ANOVA Table: Summary of All Effects (Chapter 3.2.2 *Test 1*)

Table A13.1. Three-way ANOVA: summary of all Effects

Effect	df Effect	df Error	F	p-level
F	1	24	40.5	1.0×10^{6}
L	1	24	21.6	1.0×10^{4}
C	2	24	18.9	1.2×10^{5}
F × L	1	24	36.4	3.0×10^{6}
F × C	2	24	18.6	1.3×10^{5}
L × C	2	24	5.75	9.1×10^{3}
F × L × C	2	24	6.07	7.3×10^{3}

F - Food

L - Light Regime

C - Copper concentration

Appendix 14

ASTM Feeding Tests

Aim. To measure feeding inhibition of *M. macleayi* exposed to copper in ASTM water.

Null Hypothesis. **There is no effect of copper on the feeding rate of *M. macleayi.***

Methods. Four feeding tests were performed with the following exceptions to protocols: *Test 1* used only 3 replicates per treatment, *Tests 1-3* ran for 24 h, *Tests 1-4* were conducted in a laboratory with a 12/12 light/dark regime and temperatures ranging from 27 - 31° C, and *Tests 4-5* used SEA as a food additive. Copper concentrations were: *Test1*: 0 (control), 1, 10 and 100 µg/l; *Test 2*: 0 (control),10, 18, 32, 56 and 100 µg/l; *Test 3*: 0 (control), 1, 2, 4, 7.5, 15 and 30 µg/l; *Test 4*: 0 (control), 10, 15, 20, 30, 40 and 60 µg/l; and *Test 5*: 0 (control), 10, 15, 20, 30, 60 and 100 µg/l.

Results. There was high within treatment variance all tests (see Table). Mortality was high in most tests. Power analysis performed on *Test 4* yielded that sample size of n = 7 would detect a 75 % feeding depression with power of 0.87, and n = 12 would detect a 50 % feeding depression with power of 0.84. *Tests 1-2* were invalid due to control mortality >20 %. Significant mortality occurred in many treatments (see Table A14.1), and at lower concentrations when SEA was not used. Feeding inhibition was only detected in *Test 4* when the mean (SE) feeding depression was 68 (8.7) %. This response was not confirmed when the test was repeated (*Test 5*).

Conclusion. Three observations can be made from the above results. Firstly, high and unequal variances were recorded in all tests at most treatments. Secondly, control mortality was higher when no SEA was used. Thirdly, SEA reduced the sensitivity of the animals to copper. The results are highly variable both within and between tests, and no clear response curve was apparent. It seems plausible that the erratic feeding response is due to the poor health of animals. In any case, the high control mortality rate (when no SEA was used) would undermine the validity of any response found. It is known that when cladocerans are

in optimum health, there is very little variability between life history parameters of clones under identical conditions (Baird *et al.* 1989). Therefore, more work is required to increase control survival and reduce within treatment variation. This may be achieved by an alteration of culturing conditions.

Table A14.1. Mortality and copper response data for feeding tests performed with ASTM water.

Test	**Control mortality (%)**	**Treatment mortality[#]**	**Average CV % for feeding rate treatment means**	**NOEC–LOEC (µg/l)***
1	25	100 % at 100 µg/l	125	100- >100
2	15	15 % at 18 µg/l 80 % at 32 µg/l 100 % at 56- 100 µg/l	54.9	<10-10
3	NR[+]	NR at lower treatments 100 % at 30 µg/l	23.0	2-4 (not significant at 7)
4 (SEA)	0	10 % at 10 µg/l 80 % at 60 µg/l	45.7	20-30
5 (SEA)	0	10 % at 15 µg/l 40 % at 60 µg/l 100 % at 100 µg/l	12.3	60- >60

* Nominal concentrations reported

+ Not reported

Only mortality >5 % reported

Appendix 15

Sample Volume

Aim. To investigate if sample volume size affects feeding depression.

Null Hypothesis. **There are no effects of sample volume size on feeding depression.**

Method. A 2 treatment feeding test was performed according to protocols with the exception that SEA was added to all treatments. One treatment comprised 20 ml sample volumes for all replicates; and the other treatment (control) comprised 30 ml sample volumes (i.e. normal control protocols). Feeding depressions were calculated for each treatment, and a *t*-test was performed to determine if feeding rate was affected by sample volume size.

Results. Mean (SE) feeding depression was higher for the 20 ml sample (47 (1.3) %) than the 30 ml sample (39 (5.6) %), however, this was not significant. Feeding rate was significantly higher in the 30 ml sample.

Conclusion. Feeding depression was higher and within-treatment variance lower in the 20 ml samples. However, the feeding depression was almost 50 % in this treatment, and feeding rate significantly lower than in the 30 ml treatment. This suggests that the feeding rate was possibly limited by food availability in the 20 ml treatment. It is not desirable to allow any factor that may confound measuring toxicant effects upon feeding rate, hence food limitation must be avoided. Hence, 30 ml remained the more acceptable sample size.

Appendix 16

Coulter Counter Dilution Factor

Coulter counter samples must be in an isotone solution. Algal cell counts tests performed at *eriss* using the Coulter counter routinely use a dilution factor of 1 in 2 (sample solution in isotone solution) (R. van Dam pers. comm.). Minimal variance is of utmost importance in toxicity testing in order to enable detection of small effects between treatments. It was therefore deemed necessary to establish if the Coulter counter sample dilution factor would affect the within-replicate variance.

Aim. To determine the optimal dilution factor for Coulter counter counts to provide minimum variance.

Null Hypothesis. **There is no effect of dilution factor on the variance of sample cell counts.**

Method. A solution of filtered creek water, algae and SEA was made according to usual test concentrations and methods. Three sets of Coulter counts were taken of each dilution factor: 1 in 2 (sample solution in isotone) and 1 in 4. A *t*-test was performed to determine if there was an effect of treatment on algal cell counts.

Results. There was no significant difference in algal cell counts between treatments. The coefficient of variation was lower when a 1 in 4 dilution was used (1.8, compared with 2.7).

Conclusion. There was lower within replicate variation when a 1 in 4 dilution was used, however variation in both treatments was acceptably low. The Coulter counter manual suggests measuring counts above 5000 cells per sample. As only 1 in 2 dilutions fulfilled this requirement, this dilution factor was chosen for all future testing.

Appendix 17

Clonal Variation in Response to Copper and Cadmium

Introduction. One factor favouring the use of cladocerans as test species is their mode of reproduction. Being primarily parthenogenetic organisms, variation is minimised when testing with cladocerans both within and between tests (Baird *et al.* 1991b). However, the use of clonal organisms introduces other potential problems for test reliability. Baker (1965) first coined the term 'general-purpose genotypes' in reference to broadly adapted organisms that will be favoured in harsh environments due to a general stress resistance. It has been suggested that clonal selection for general-purpose genotypes is easier amongst parthenogenetic than sexual organisms, hence explaining their wide distribution in marginal habitats (Forbes *et al.* 1997). Clones that have general stress resistance are likely to be amenable to laboratory culturing and hence become relied upon for testing. Yet they may not always be representative of their species, family or trophic level, in terms of sensitivity to toxicants.

Investigation of cladoceran clonal variation in response to toxicants has also yielded the 'essentiality hypothesis' (Barata *et al.* 1998) - a suggestion that there may be general response mechanisms which respond to essential metal toxic stress, and that more specific mechanisms are responsible for organisms enduring non-essential metal toxicity. This hypothesis is in response to findings that some *D. magna* clones exhibit higher variation in response to trace metals than to micronutrients (Baird *et al.* 1991b). This is an area of research important to investigate, not only because it brings into question the validity of single clone tests. These findings also suggest different modes of action between particularly essential and non-essential metals, and also support the possibility of induction of metal tolerance (Stuhlbacher *et al.* 1992).

Aim. To measure clonal variation in response to copper and cadmium toxicity, using 3 different clones of *M. macleayi.*

Null Hypothesis. **There are no differences in responses between clones when exposed to copper and cadmium.**

Alternative Hypotheses.

1. There are greater variations in response between clones to a non-essential (i.e. cadmium) than essential (i.e. copper) metal (cf. the 'essentiality hypothesis' - Barata *et al.* 1998).
2. Clones collected from waters with elevated metal concentrations exhibit lower sensitivity in response to metal exposure than other clones ('induction of tolerance' hypothesis - Stuhlbacher 1992).
3. Clones cultured in a laboratory for an extended period exhibit lower sensitivity in response to metal exposure than clones collected from the wild ('general purpose genotype' - Baker 1965).

Material and Methods.

The 3 clones were chosen for testing based on their markedly different past environmental conditions. All clones were originally from local waterbodies, however, 1 clone had been collected from Magela Creek floodplain and cultured in the *eriss* laboratory for over 10 y.

One of the 'wild' clones was collected from Bowerbird Billabong (BB) (Arnhem Land) on 15/10/98 (pH: 5.6; Conductivity: 18 µS/cm; Dissolved Oxygen: 88 %; Alkalinity: 5.6 mg/l). BB is seasonally connected to Magela Creek, and approximately 18 km north-east (upstream) of Jabiru. During the dry season, culturing and test dilution water is collected from BB as it is considered a pristine waterbody. The second wild clone was collected from Ranger Retention Pond 1 (RP1) on 25/9/98 (pH: 7.3; Conductivity: 220 µS/cm; Dissolved Oxygen: 83 %; Alkalinity: 56 mg/l). RP1 is on the Ranger uranium mine-site and is designed to retain the excess water that falls on the least contaminated areas of the mine-site during the wet

season. It is considered to have slight metal contamination, in particular, uranium (<0.1-2 µg/l), manganese (1-20 µg/l) and zinc (<2-26 µg/l) (ERA 1998).

Both wild clones were collected from shallow reedy areas with a sweep net. *M. macleayi* was identified from Julli (1986). As filtered Magela Creek (BB) water is used for animal culturing, the BB clone were moved immediately to this water. The RP1 clone were placed in a bowl of 50/50 % filtered Magela Creek-RP1 water for 48 h, then 75/25 % for 48 h, then on the fifth day, were placed in 100% filtered Magela Creek water. Both clones were cultured in 1.5 l covered and aerated glass bowls in a 27±1 °C, 12/12 light/dark incubator. Every Monday, Wednesday and Friday, animals were removed with an eyedropper and placed in a clean bowl with 1.5 l filtered Magela Creek water, with *Chlorella* sp. (2×10^5 cells/ml) and FFV (1 ml/l). At least 3 weeks prior to testing, females were removed to individual cultures (according to *eriss* protocols for production of test animals (Hyne *et al.* 1996)) and at least 4 healthy generations (of second brood females) were reared in this manner until testing commenced.

The endpoint of feeding inhibition was found to be a sensitive indicator of toxicity for cadmium, but not for copper (Chapter 5). Therefore, reproduction bioassays were used for measuring response to copper, and feeding bioassays were used for measuring response to cadmium.

Copper. Three reproduction bioassays were performed commencing on the dates 15/11/98, 3/11/98 and 30/11/98 on the laboratory (Lab) (Chapter 5.2.1 *Test 5*), RP1 and BB clones, respectively. Copper concentrations were: 0 (control), 10, 20, 25, 30 and 35 µg/l.

Cadmium. One feeding bioassay was performed on each of the RP1 and Bowerbird Billabong (BB) clones. Two feeding tests were performed on the laboratory (Lab) (Chapter 5.3.2 *Tests 4-5*). The cadmium concentrations used for the 2 wild clones were: 0 (control), 0.5, 1, 1.7, 3, 5.5, 10 and 18 µg/l.

Results.

Copper. The lab clone showed the highest sensitivity to copper, followed by the RP1 clone, and then the BB clone (Table A17.1). All 2 clones were significantly different from each other at EC5; at EC20, only the Lab clone was significantly different from the BB clone.

Cadmium. In the BB clone feeding test, mortality was ≥20 % in all treatments ≥3 µg/l, and 10 % in 0.5 µg/l and controls. Despite this, animals in treatments 5.5 and 18 µg/l showed no feeding inhibition. There was no significant difference in any point estimate between any clone (Table A17.2).

Table A17.1. Effect of copper on reproduction for 3 clones of *M. macleayi*; results from hypothesis testing and point estimates.

Clone	**NOEC- LOEC** (µg/l)	**EC5 (95 % CI)** (µg/l)	**EC20 (95 % CI)** (µg/l)
Lab clone	9.2- 19	2.6 (1.7- 7.9)	9.4 (5.7- 17)
RP1 clone	18- 23	15 (3.2- 18)	19 (13- 19)
BB clone	23- 29	20 (4.8- 21)	21 (20- 24)

Table A17.2. Effect of cadmium on feeding rate for 3 clones of *M. macleayi*; results from hypothesis testing and point estimates.

Clone	**NOEC- LOEC** (µg/l)	**EC5 (95 % CI)** (µg/l)	**EC20 (95 % CI)** (µg/l)
Lab clone (Ch 5.3.2 *Test 4*)	0.52- 1.0	(0.2 - 1.1)	8.4 (na)
Lab clone (Ch 5.3.2 *Test 5*)	1.2- 1.8	(0.0 - 0.8)	5.7 (0.0 - 7.5)
RP1 clone	1.1- 1.7	(0.1 - 1.3)	3.4 (0.80- 6.2)
BB clone	0.78- 1.3*	(0.0 - 0.71)	6.6 (na)

* No significant effect at 5.7 and 19 µg/l.

Conclusion.

Although only few tests have been performed for each metal, results do not support any of the alternative hypotheses suggested. Surprisingly, the only sign of variations in clonal response, occurred for copper, and these results inferred that the laboratory clone was the most sensitive clone, and the BB clone the least sensitive. However, the differences were not great, hence may disappear with further replication. At this stage of testing, there is no support for the 'essentiality hypothesis' (Barata *et al.* 1998), or the general purpose genotype hypothesis (Baker 1965). The lower sensitivity to copper detected in the wild clones may be the result of induction of tolerance (Stuhlbacher 1992) in natural waters.

Appendix 18

Production of Fermented Cichlid Food Additive (FFV) (Hyne *et al.* 1993)

Five grams of pelleted cichlid food (Wardley Products, New Jersey), and 0.25 g of dried and powdered alfalfa are homogenised in 250 ml of Milli-Q water for 5 minutes using a domestic food-blender. The mixture is then bubble-aerated for 3 d at ambient water temperature (25-28° C), covered with a fine mesh to ensure it does not become contaminated by flies etc., and to allow any gases to escape. At the end of this 3 d period the mixture is then collected into a beaker and placed at 4° C for 1 h and allowed to settle. The supernatant (150 ml) is then decanted carefully into another beaker. This is then divided into aliquots of about 1 ml in glass vials, capped and frozen. An aliquot is then thawed when needed and resuspended by gentle shaking before use. The aliquot can then be refrigerated (4° C), but is discarded 1 week after thawing. Addition to *M. macleayi* culturing and testing solution is 1 µl/ ml.

Appendix 19

Gold Mine Water Chemical Analysis

Table A19.1. Analysis of gold mine release water chemical composition (Temperature: 31 °C; pH: 3.0) .

Chemical	Measured amount
Ca	89 mg/l
Mg	89 mg/l
Na	14 mg/l
K	7.1 mg/l
SO_4	847 mg/l
Cl	196 mg/l
NO_2	NR[1]
NO_3	NR
SiO_2	67 mg/l
Al-(filtered)	28728 µg/l
Al-(total)	28620 µg/l
As-(filtered)	<1 µg/l
As-(total)	<1 µg/l
Bi-(filtered)	<0.2 µg/l
Bi-(total)	<0.2 µg/l
Cd-(filtered)	139 µg/l
Cd-(total)	141 µg/l
Co-(filtered)	333 µg/l
Co-(total)	326 µg/l
Cr-(filtered)	3 µg/l
Cr-(total)	3 µg/l
Cu-(filtered)	617 µg/l
Cu-(total)	603 µg/l
Fe-(filtered)	5725 µg/l
Fe-(total)	5755 µg/l
Mn-(filtered)	4050 µg/l
Mn-(total)	4350 µg/l
Ni-(filtered)	1375 µg/l
Ni-(total)	1478 µg/l
Pb-(filtered)	17 µg/l
Pb-(total)	15 µg/l

Chemical	Measured amount
Sb-(filtered)	<0.2 µg/l
Sb-(total)	<0.2 µg/l
Se-(filtered)	<5 µg/l
Se-(total)	<5 µg/l
U-(filtered)	29 µg/l
U-(total)	28 µg/l
Zn-(filtered)	6350 µg/l
Zn-(total)	7388 µg/l

[1] Not reported