internal report **369**

Development and application of the Moinodaphnia macleayi feeding rate bioassay for rapid toxicity assessment

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supervising scientist

Development and application of the *Moinodaphnia macleayi* feeding rate bioassay for rapid toxicity assessment.

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Research thesis for Bachelors of Applied Science (Honours)

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List of Abbreviations:

NOEC	no observed effect concentration
LOEC	lowest observed effect concentration
$EC_{5/20/50}$	effective concentration 5, 20 or 50% of the organisms relative to control
$T_{0/20/40}$	time = 0, 20, 40 hours
eriss	Environmental Research Institute of the Supervising Scientist
NT	Northern Territory
ARR	Alligator Rivers Region
KNP	Kakadu National Park
ERA	Energy Resources of Australia
RP2	retention pond 2
RP4	retention pond 4
OECD	organisation for Economic Co-operation and Development
CB	Coulter blank
Cd	cadmium
FFV	fermented fish food with vitamins
SD	standard deviation
95% CI	ninety five percent confidence interval
ANOVA	analysis of variance
h	hour
°C	degrees Celsius
ppm	parts per million
ppb	parts per billion
mm	millimetre
cells/mL	algal cells per millilitre
µg/L	micrograms per litre
mg/L	milligrams per litre
µS/cm	micro siemens per centimetre
MBAS	methyl blue active substances
CTAS	cobalt thiocyanate active substances
LAS	linear alkylbenzene sulphonates
AES	alkyl ethoxylated sulphates
WET	whole effluent testing
DTA	direct toxicity assessment
ATP	adenosine tri-phosphate
DOM	dissolved organic matter

Chapter 1: Introduction

1.1 Background to ecotoxicology

In recent years increased development of industry, agriculture and urban centres has applied a significant amount of pressure on Australia's wetlands and aquatic ecosystems (Bunn *et al.*, 1997). This expansion has also brought about an increase in the production of domestic and industrial waste waters (van Dam *et al.*, 1998). Effluents from mining and ore processing, agricultural runoff and domestic wastes such as sewage, are all potentially toxic to aquatic organisms and may have long term effects on receiving waters (Chapman, 1995a). In Australia, ecotoxicology has only recently gained recognition as an essential tool for environmental impact assessment of chemicals and complex wastes (ASTEC, 1991; In Chapman, 1995a). Ecotoxicology is an interdisciplinary science combining aspects of physiology, biochemistry, histology and environmental chemistry (Rand *et al.*, 1995), to assess the effects of chemicals on a range of species and on inter-species interactions (Chapman, 1995a). The impact of toxicants on aquatic ecosystems can be estimated using ecotoxicological bioassays to measure the toxicity of single chemicals or effluents (M.B.L, 1999).

1.1.1 The application of toxicity tests

Ecotoxicological testing provides a knowledge base from which the impact of chemicals and effluents on receiving waters can be predicted (Chapman, 1995b). They are also useful to screen and rank chemicals to predict their hazard and risk, determine cause-effect relationships in post-impact studies and to determine dilution coefficients of effluents prior to discharge (Chapman, 1995b). In principle, toxicity tests can be carried out at any level of organisation ranging from sub-cellular through to ecosystem (Buikema Jr *et al.*, 1982). As systems become less complex (i.e. ecosystem to molecular) variables become easier to control but relevance to ecosystems decreases (Calow, 1993). Population and community levels are particularly difficult to assess in the laboratory and

most tests are conducted at a 'whole organism level' (Calow, 1993; Holdway *et al.*, 1988). Mortality and sub-lethal effects are assessed in the laboratory using a range of standardised acute and chronic methods (Cooney, 1995).

1.1.1.1 Acute tests

Acute tests are short term, have a simple experimental design and usually measure mortality or immobilisation as an endpoint (Cooney, 1995). Information about the lethality of a chemical or waste water is obtained by determining the highest concentration of the material which affects a certain percentage of a population over a certain time period (e.g. 50% for 24 or 48 hours; Rand *et al.*, 1995). Toxicity testing using acute endpoints is simple, cost effective, ecologically relevant and scientifically and legally defensible (Buikema Jr *et al.*, 1982). However, one major downfall is that a number of sub-lethal responses such as growth, reproduction, feeding and fecundity are overlooked.

1.1.1.2 Chronic tests

The term 'chronic' refers to the duration of the test and is usually associated with exposures conducted over more than 10% of an organism's life span (Cooney, 1995). In general, organisms are exposed to a series of concentrations of a toxicant, to determine its effects on life stages and essential processes (Buikema Jr *et al.*, 1982). The time period of the test is directly proportional to the organism's life cycle (weeks, months or years). Even though chronic toxicity tests are more complex and more expensive to run and information produced is more effective for predicting concentrations not likely to harm a population (*i.e.* "safe" concentrations; Buikema Jr *et al.*, 1982). Types of chronic tests vary from short term to full life cycle tests and measure an assortment of biological endpoints including mortality, reproductive impairment, immobilisation, impairment of growth, enzyme activity, inhibition of bioluminescence, swimming rate, motility, ventilation rates and feeding rates (Rand *et al.*, 1995). Flickinger *et al.* (1982) stated that

bioassays evaluating only survival and reproduction may underestimate toxicity and therefore behavioral changes are more appropriate, as rapid and sensitive indicators of toxic stress. Previous studies have found this to be the case in fish (Sprague *et al.*, 1965; Bengtsson, 1974; Besch *et al.*, 1977; In Flickinger *et al.*, 1982), however, there has been little work developing behavioural endpoints in other taxa.

1.1.2 Single chemical versus whole effluent toxicity testing

The toxicity of complex mixtures is extremely difficult to anticipate. An individual chemical-by-chemical approach would be time consuming, costly, and would not take into account interactions between chemicals. Even after there has been considerable effort into analysis, a sound assessment cannot be conducted due to difficulties relating results to ecological impact (Chapman, 1995a). Therefore, whole effluent toxicity (WET) testing or direct toxicity assessment (DTA) is used to assess the effects of combined toxicity, which cannot be demonstrated from exposure to the pollutants separately (Witters, 1998). DTA conducted as acute or short term chronic tests, is an effective tool for estimating potential effects of effluent discharges into aquatic systems (Parkhurst *et al.*, 1992). It is a more direct test which not only measures the toxic response but also the interaction of the toxicants with other constituents within the sample (M.B.L., 1999). Although DTA is not able to identify the components of a mixture (Jop *et al.*, 1991; In van Dam and Chapman, 2000) it is a useful measure of toxicity and bioavailability, leading to more accurate prediction of impacts on communities (Waller *et al.*, 1996; In van Dam and Chapman, 2000).

1.2 Ecotoxicology in the Australian Wet-Dry tropics

1.2.1 History

Research into the effects of mine contaminants on aquatic systems in the wet-dry tropics of Australia was initiated in 1982 by the Office of the Supervising Scientist (OSS), currently the Environmental Research Institute of the Supervising Scientist (*eriss*). In

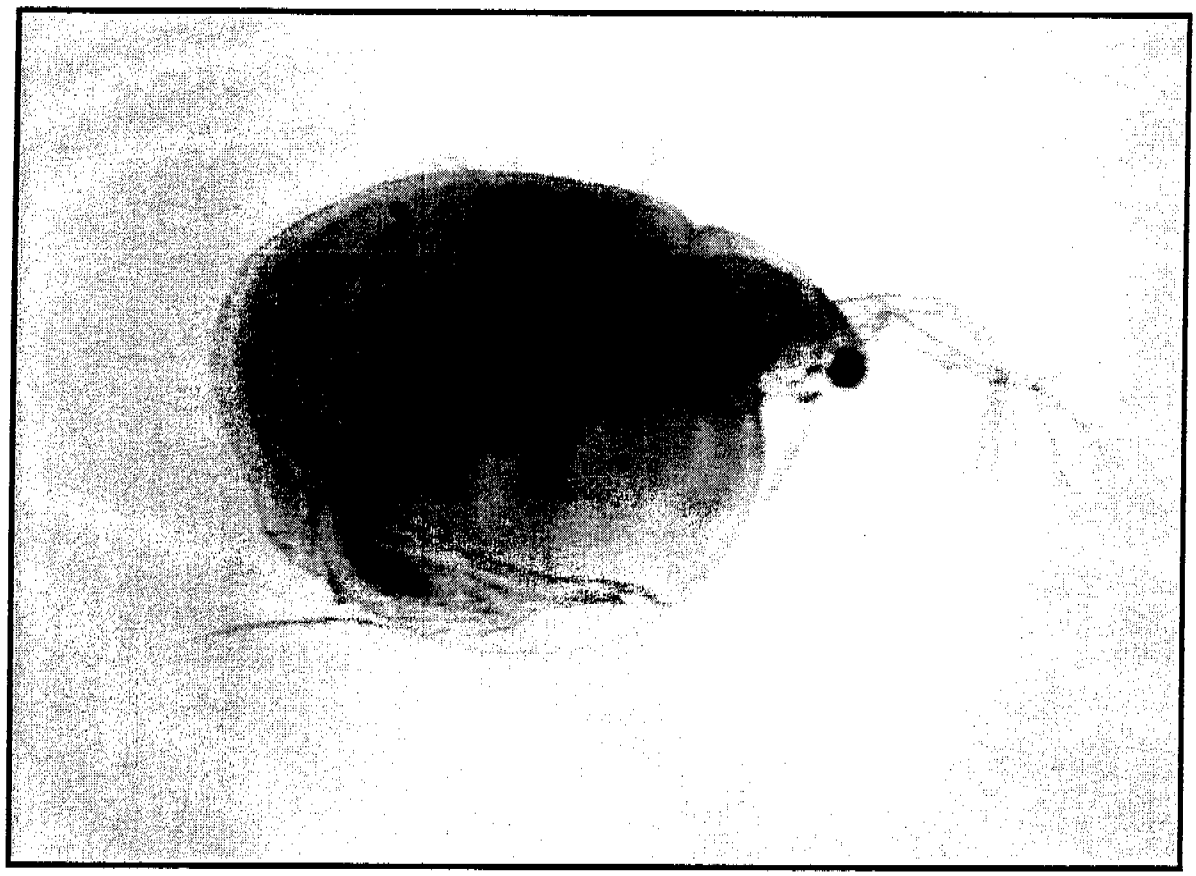
early years the program was primarily concerned with the effects of single chemicals such as zinc, lead, and copper. The development of an extensive direct toxicity assessment program then enabled accurate monitoring of pre-release waste waters from the Ranger Uranium Mine (ANZECC, 2000). Routine testing is currently undertaken on tropical test species of fish, algae and Cladocera. Cladocerans comprise a major component of freshwater zooplankton in numbers and biomass, providing a principal food source for most fish and larger organisms (Ravera, 1991). Their abundance in natural systems, ease of culture and short generation time has favoured them for study by limnologists and population ecologists worldwide (Table 1.1)(Herbert, 1978). Of the many organisms found in freshwater environments, crustacean zooplankton have demonstrated increased sensitivity to heavy metals (Wong, 1993). This susceptibility has encouraged scientists to use cladocerans as indicators of water pollution and for the management of industrial effluents (Tomasik *et al.*, 1995).

1.2.2 The tropical cladoceran, *Moinodaphnia macleayi*

Microcrustaceans are a crucial component of the aquatic ecosystems in the Australian wet-dry tropics, with 43 species of Cladocera documented in the Magela Creek flood plain (Kakadu National Park) alone. Therefore, cladocerans are appropriate organisms for biological testing purposes (Julli, 1986). Of 19 aquatic species assessed by Holdway (1992a) no one species was always the most sensitive, although *Hydra* spp., larval Purple Spotted Gudgeon (*Mogurnda mogurnda*) and the tropical cladoceran, *Moinodaphnia macleayi,* tended to be consistently more sensitive than other species in the group (Holdway, 1992a). *M. macleayi* is found in weed-bed habitats of coastal Northern Territory, New South Wales and Queensland. It has a short reproductive cycle and is small relative to other Cladocera, with adult females measuring about 1mm in diameter (Figure 1.1)(Julli, 1986). For these reasons *M. macleayi* has been used in the continuing development of pre-release screening techniques and post-release assessment of waste waters (Hyne *et al.*, 1996; Camilleri *et al.*, 1998; van Dam *et al.*, 1999).

Table 1.1: Advantages and disadvantages of using Cladocerans as test organisms

Advantages	Source	**Disadvantages**	Source
Small size – culture is easy and inexpensive	Baudo, 1987	Unfed animals cannot be used in tests lasting more than 48 h	Adema, 1978
Sensitivity to toxicants	Wong, 1993	Lack of test reproducibility	Baudo, 1987
High fecundity	Kovisto, 1995	Toxicant sensitivity is age dependent	Baudo, 1987
Parthenogenetic reproduction (uniformity of response)	Julli, 1986	Considerable growth may confuse results in long term tests	Baudo, 1987
Short life span and reproductive cycle	Baudo, 1987		



1mm

Plate 1.1: Photograph of the tropical Cladoceran *Moinodaphnia macleayi* (Female, Lateral View)

1.2.3 Metals of ecotoxicological concern

1.2.3.1 Sources

There are approximately 20 different mining sites in the Northern Territory of Australia, with a significant amount of these located adjacent to wetland areas and floodplain catchments (DME, 2000). Substances extracted include metallic minerals such as gold, manganese, copper, lead and zinc; industrial minerals such as diamonds, vermiculite, salt, gravel and energy minerals such as uranium (DME, 2000). Those of most ecotoxicological concern in the wet-dry tropics are uranium, manganese, zinc (Holdway, 1992b), copper (Markich and Camilleri, 1997) aluminium (van Dam *et al.*, 1999) and cadmium. These metals have been studied extensively in Cladocera (Table 1.2). Complex wastes of concern in the area consist of various combinations of these metals, petroleum products, organic chemicals (Holdway, 1992a), treated sewage and domestic waste (PAWA, 1999).

1.2.3.2 Metal speciation and mechanisms of toxicity

Both inorganic and organic substances are known to alter feeding response in *Daphnia* and related species. Filtering rates, ingestion, digestion and assimilation have all been studied in much detail, however few studies have outlined the mechanisms underlying feeding inhibition (Allen *et al.*, 1995). In general, metals exert a toxic effect on animals when they enter biochemical reactions where they are not normally involved (Depledge *et al.*, 1994). This interference may result in damage to plasma membranes due to binding to phospholipids and proteins, inhibition of Na/K-dependent ATPases, inhibition of transmembrane amino acid transport, lipid peroxidation, enzyme inhibition and depletion

Table 1.2: Metal toxicity data for Cladoceran species

Metal	Organism	Life Stage	Water Hardness (mg/L $CaCO_3$)	pH	Test Duration	Endpoint	Toxic Concentration (µg/L)	Source
Cd	*D. magna*	12h	45.3	NR	48h	Mortality	LC50 @ 65	Beisinger & Christensen, 1972
	D. magna	NR	45.3	NR	21d	16% Reproductive Impairment	0.17	Beisinger & Christensen, 1972
	D. magna	14d	150	8.4	14d	Consumption Rate	EC60 @ 5	Bodar *et al.*, 1982a
	D. magna	~12 h	150	8.4	8-9d	Mortality	LC50 @ 10	Bodar *et al.*, 1982b
	M. macleayi	>6h	NR	6.5	5d	Reproduction	NOEC-LOEC (1.0-1.9)	Semaan, 1999
	D. pulex	≤24h	80-90	NR	48h	Mortality	LC50 @ 78	Roux *et al.*, 1993
Mg	*D. magna*	12h	45.3	NR	48h	Mortality	LC50 @ 140000	Biesinger & Christensen, 1972
	D. magna	12h	45.3	NR	21d	16% Reproductive Impairment	82000	Biesinger & Christensen, 1972
Mn	*D. magna*	12h	45.3	NR	48h	Mortality	LC50 @ 9800	Biesinger & Christensen, 1972
	D. magna	<24h	330	8.0	8h	Accumulation	Maximum uptake (CF = 65) @ 8h	Kwasnik *et al.*, 1978
Zn	*D. magna*	12h	45.3	NR	48h	Mortality	LC50 @ 100	Biesinger & Christensen, 1972
	D.magna	12h	45.3	NR	21d	16% Reproductive Impairment	4100	Biesinger & Christensen, 1972
	M. macrocopa	>24h	NR	6.5	10d	Significant Increase in Mortality	500	Wong, 1993

Table 1.2 Continued

Metal	Organism	Life Stage	Water Hardness (mg/L $CaCO_3$)	pH	Test Duration	Endpoint	Toxic Concentration (µg/L)	Source
Al	*D. magna*	12h	45.3	NR	48h	Mortality	LC50 @ 3900	Biesinger & Christensen, 1972
	D. magna	adult	2.5	5.0	24h	Loss of Movement	EC50 @ 1020	Havas, 1985
	D. magna	12h	45.3	NR	21d	16% Reproductive Impairment	320	Biesinger & Christensen, 1972
	D.pulex	12h	130-160	NR		~80% Reproductive Impairment	70	Winner & Farrell, 1976
	C. sphaericus	adult	33.8	8.4	48h	Mortality	EC50 @ 7.6	Kovisto *et al.*, 1992
U	*M. macleayi*	<6h	4.8	6.6	24h	Mortality	LC50 @ 1470	Bywater *et al.*, 1991
	D. macrops	<6h	4.8	6.6	24h	Mortality	LC50 @ 1254	Bywater *et al.*, 1991
	D. excisum	<6h	4.8	6.6	24h	Mortality	LC50 @ 1140	Bywater *et al.*, 1991
	L. fasciculata	<6h	4.8	6.6	24h	Mortality	LC50 @ 467	Bywater *et al.*, 1991
	M. macleayi	<6h	4	6.5	48h	Mortality	LC50 @ 211	Markich & Camilleri, 1997
	M.macleayi	adult	NR	6.5	24h	Mortality	EC50 @ 160	Semaan, 1999

Table 1.2 Continued

Metal	Organism	Life Stage	Water Hardness (mg/L $CaCO_3$)	pH	Test Duration	Endpoint	Toxic Concentration (µg/L)	Source
U	*M.macleayi*	<6h	NR	6.2	5d	Reproduction	NOEC-LOEC (1-3.2%)	Markich & Camilleri, 1997
	M. macleayi	<6h	NR	6.5	5d	Reproduction	NOEC-LOEC (7.8-20.0)	Semaan, 1999
	M. macleayi	adult	NR	6.2	24h	Feeding	NOEC-LOEC (0.3-1%)	Orchard, 1999
	M. macleayi	<6h	4	6.5	5d	Reproduction	LOEC @ 22	Markich & Camilleri, 1997
Cu	*D. magna*	adult	250	NR	7d	Growth and Survival	6.6	Dave, 1984
	D. magna	6-24h	250	NR	48h	Mortality	LC50 @ 18.5	Dave, 1984
	M. macrocopa	>24h	NR	6.5	48h	50% Reduction in Life-Span	150	Wong, 1993
	D. pulex	≤24h	80-90	NR	48h	Mortality	LC50 @ 21	Roux *et al.*, 1993
	D. magna	12h	130-160	NR	72h	Mortality	LC50 @ 86.5	Winner & Farrell, 1976
	D. pulex	12h	130-160	NR	72h	Mortality	LC50 @ 86	Winner & Farrell, 1976
	D. magna	12h	45.3	NR	48h	Mortality	LC50 @ 9.8	Biesinger & Christensen, 1972

of reduced glutathione (Viarengo, 1989; In Depledge *et al.*, 1994). In invertebrates these molecular changes may result in suppression of growth, impaired reproduction, impairment of tissue repair systems, supression of oxygen consumption and affect ecdysis (Viarengo, 1989; In Depledge *et al.*, 1994). The toxicity of metals is commonly related to their chemical form and their relative ability to bind to organic ligands (Mason and Jenkins, 1995). The toxicity of class B metals such as cadmium and copper is significantly increased by the lack of binding specificity to organic ligands, resulting in a greater probability of non-specific binding to molecules within the body. Other studies have also attempted to correlate physicochemical properties (e.g. pH, hardness and dissolved oxygen) to the modes of action for particular metals. However, this approach has not been without problems mainly due to the differences between essential and non-essential metals (Mason and Jenkins, 1995). Essential metals are actively involved in life processes as structural components, enzymatic proteins or in the maintenance of electrolyte balance, while non-essential metals have no known biological function. It is therefore more probable that toxicity is related more to demand and use rather than physicochemical properties (Mason and Jenkins, 1995). Barata *et al.* (1998) supported this theory by demonstrating that acute responses are more variable in non-essential metals than essential metals. Essential metals occur in high amounts within biota and therefore are subject to continuous directional selection, where as non-essential metals are only acquired by intermittent selection (Barata *et al.*, 1998).

1.3 Rapid toxicity bioassays

The estimation of mining effluent toxicity can be confused due to a range of synergistic and antagonistic interactions (Jansen and Persoone, 1993). To eliminate problems such as lengthy test duration and expense, more rapid, realistic and reliable methods have been developed. These methods may assist with detection of wetland degradation and act as a warning system for aquatic environments (van Dam *et al.*, 1998). Whilst chronic toxicity tests have proven to be sensitive and reliable indicators of toxicity and provide a good

indication of potential population effects, they are not particularly rapid to assess. Lengthy test durations are not favourable for developing "safe" dilution coefficients for the immediate release of toxic wastewater from mine sites or to provide water managers with rapid answers to take action after a chemical spill. Such situations require a rapid test (≤24h) that has demonstrated sensitivity and ecological relevance. Some examples of rapid toxicity tests are discussed below.

1.3.1 Bacteria

Various types of bacterial bioassays have been developed to meet the above criteria, including Microtox® and modified versions (Thomulka and Lange, 1997), luminescent *Escherishia coli* and *Salmonella* assays (Belkin *et al.*, 1996) and mutagenic assays such as Mutatox. Of these, Microtox® is the most commonly used. It is based on measurable changes in light production by the luminescent marine bacterium *Vibrio fisheri* or *Photobacterium phosphoreum* when exposed to toxicants (Retina *et al.*, 1989; van Dam *et al.*, 1998). While Microtox® is a simple, rapid and cost-effective bioassay, the need for osmotic adjustment and dilution eliminates the possibility of testing full-strength effluents (Stauber *et al.*, 1994). There is also a need to investigate microbial processes in unimpacted systems to ascertain whether bacterial bioassays carry sufficient ecological relevance to accurately determine impacts to freshwater aquatic systems.

1.3.2 Algae

Over the past two decades the use of algal species in toxicity tests and as indicators of environmental change has notably increased. Unicellular algae are the basis of production in marine and lentic systems and play an important role in food chains in most aquatic systems (Franklin *et al.*, 1998). Alongside their aquatic importance, algal tests are relatively inexpensive, rapid and sensitive (McCormick and Cairns, 1994; Stauber, 1995; van Dam *et al.*, 1998). Algal bioassays undertaken in a laboratory have the potential to

assess a range of functional endpoints. Even though population growth and cell division are more sensitive, respiration, fluorescence, ^{14}C uptake and enzyme activity are more rapid assessments due to a shorter incubation period (Stauber, 1995). Although algal bioassays are useful tools for assessing the impact of certain chemicals on individual species, this approach is not representative of natural systems (van Dam *et al.*, 1998). This problem has been partially overcome in the laboratory by using natural assemblages of algae and including principal environmental factors or by conducting the experiments *in situ* (van Dam *et al.*, 1998).

1.4 Feeding rate as a rapid endpoint

1.4.1 Feeding rate bioassays

There have been several methods developed for the study of feeding in daphnids. Wong *et al.* (1983) observed the feeding behaviour of *Daphnia pulex* by dorsally tethering the animal and exposing it to crude oil particles. Even though metabolism tended to increase, there was significant disruption to feeding patterns. While this study was reasonably effective in measuring filtering rates, it is impractical, as tethering organisms restricts their movement having a significant effect on filtering rates (Jones *et al.*, 1991). Other methods include measuring the consumption rate of a fluorescent stained food source. In this case toxicity is determined by the presence of fluorescence in the gut using image analysis (Bitton *et al.*, 1996; Juchelka and Snell, 1995). Juchelka and Snell (1995) achieved results that correlated with reproductive tests for a number of toxicants, while CerioFAST®, a test developed by Bitton *et al.* (1996), achieved results similar to those of the 48-hour acute bioassay. There were however some problems associated with these methods; manipulation of the test organisms both before and after feeding brought about undue stress to the animals, while feeding regimes and food type were not realistic. Other tests using a more genuine food source, have attained more realistic results for feeding, metal adsorption and desorption in the gut. In this case feeding rate is determined by calculating changes in ambient algal cell concentrations, using an electronic particle

counter. This method has proven to be effective, easy and sensitive, favouring its use in further experiments (Allen *et al.*, 1995).

1.4.2 Effects of age on toxicant sensitivity

The juvenile stage of the organism's life cycle is generally the most sensitive. Therefore, it is possible to develop cost-effective, short term tests to replace the existing chronic tests by concentrating tests on this life stage (Baird *et al.*, 1991). In *Daphnia* spp., neonates (≤ 24 hours) are used for both acute and chronic tests (Cooney, 1995). Cooney (1992b) conducted tests with NaCl, using three different age groups of *C. dubia* (0-4, 20-24, 0-24h), to determine whether age at initiation of the test had a significant effect on test results. There was evidence of reproductive implications with the younger age group (0-4h) having fewer young per adult than the older age group (20-24h). One possible explanation for this is that the older age group were producing their fourth brood at the end of the test whereas the younger age group were only just producing their third (Cooney *et al.*, 1992b). It is also important to note that *M. macleayi* is a smaller organism than most *Daphnia* species, with a correspondingly shorter life span and therefore brood times are considerably different.

1.4.3 Effects of crowding on feeding rate

In natural systems densities of zooplankton populations are well below those used in laboratory experiments (Hanson and Peters, 1984; In Peters, 1987). *M. macleayi* is a small species relative to other *Daphnia* spp. and therefore more organisms are required to be able to measure feeding rate (Orchard, 1999). Consequently the influence of crowding on cladocerans may result in aggressive behaviour and stress due to increased concentrations of metabolic wastes (Cooney, 1995) and competition for food (van Leeuwen *et al.*, 1985). It is also known that depletion of resources in the test system can effect both survival and reproduction by reducing the foraging efficiency and increasing the filtering rate (Kersting, 1978; In van Leeuwen *et al.*, 1985).

1.4.4 Effects of toxicant exposure on feeding rate

Both inorganic and organic chemicals have been found to alter cladoceran feeding rate, resulting in an overall reduction in filtering rates and processing (*i.e.* ingestion and digestion). Positively charged species are more likely to adsorb to negatively charged surfaces (e.g. algal cells) and will therefore have a higher likelihood of interfering with feeding (Allen *et al.*, 1995). This principle was investigated when cadmium (Cd^{2+}) was exposed to the freshwater alga, *Chlorella vulgaris*. A comparison of the effects of algal-bound Cd^{2+} and dissolved Cd^{2+} demonstrated that the inhibition of feeding parameters in *Daphnia magna* was almost completely due to surface binding, with the exception of lethal concentrations, where algal cells actually reduced the toxicity (Taylor *et al.*, 1998). Similarly, filtration and ingestion rates of *D. magna* decreased with increasing concentrations of tetradifon (4-chlorophenol 2,4,5-trichlorophenol sulphone) following short-term exposure. Studies have found that cadmium may cause feeding effects by:

i) Damage to feeding apparatus upon contact with cadmium contaminated food particles.
ii) Reduced absorption in the gut allowing cells to pass through undigested and/or
iii) Rejection of contaminated food due to 'taste' (Taylor *et al.*, 1998; Allen *et al.*, 1995).

Support for all three hypotheses can be found in the literature, however, Allen *et al.* (1995) suggested that the likely mechanisms are damage to feeding apparatus and rejection due to 'taste'.

As a result of its strong tendencies to inhibit feeding, cadmium was used for the development of a feeding rate bioassay using *M. macleayi* (Orchard, 1999). This test was not a suitable indicator of toxicant sensitivity for copper, uranium and retention pond 4 water from Ranger Uranium Mine, however, a strong feeding inhibition was observed at low cadmium levels and in tests using gold mine release water (Orchard, 1999). Thus, it

was concluded that the *M. macleayi* feeding test had potential application in toxicity assessment but required further development. This would entail the investigation of factors such as food concentration and quality, body size, temperature, diel rhythms, crowding, pH and oxygen (Lampert, 1987; In Sterner and Smith, 1993; Orchard, 1999).

1.5 Aims

General aims of this research were:

1. To validate the initial feeding test protocol developed by Orchard (1999)

2. Further refine this protocol to increase sensitivity, reliability and cost-effectiveness of the test

3. Assess the ability of the final feeding test protocol to estimate toxicity for a range of complex wastes.

Chapter 2: General Materials and Methods

2.1 Site description

All research was undertaken at the Environmental Research Institute of the Supervising Scientist (*eriss*) ecotoxicology laboratory between April and September 2000. The laboratory is situated on the Ranger Uranium Mine Pty. Ltd. mineral lease at Jabiru East NT, surrounded by Kakadu National Park.

2.2 Diluent water

Diluent water, for maintaining stock cultures and testing purposes, was collected six times over the testing period from one of two sites along the Magela Creek, depending on seasonal availability. In the Dry season (June to October) water was collected from Bowerbird Billabong, West Arnhem Land (Figure 2.1), and during the Wet season (November to May) water was collected at Georgetown Billabong (Figure 2.2). Bowerbird billabong is located apporximately 18 km upstream of Georgetown Billabong (Figure 2.3). Water was filtered through 2.5µm filter paper (Whatman No. 42) to remove zooplankton and particulate matter, and refrigerated at 4 °C in five litre polyethylene screw top bottles or 25 litre polyethylene barrels. Dissolved oxygen, pH and electrical conductivity were recorded both before and after filtration (Appendix 3). Background metal concentrations for both sites are displayed in Table 2.1, and except for of a slightly higher background uranium concentration in Bowerbird billabong water, there are no significant differences in water quality between sites.

2.3 Maintenance of stock cultures

Laboratory stock of *M. macleayi* was maintained at *eriss* in a constant temperature incubator at 27 °C using one animal per vial, a light regime of 12 h light: 12 h dark. Each 45 mL vial had a screw cap with two, 2 mm diameter ventilation holes and contained 30

Figure 2.1: Photograph of Bowerbird Billabong, August 2000

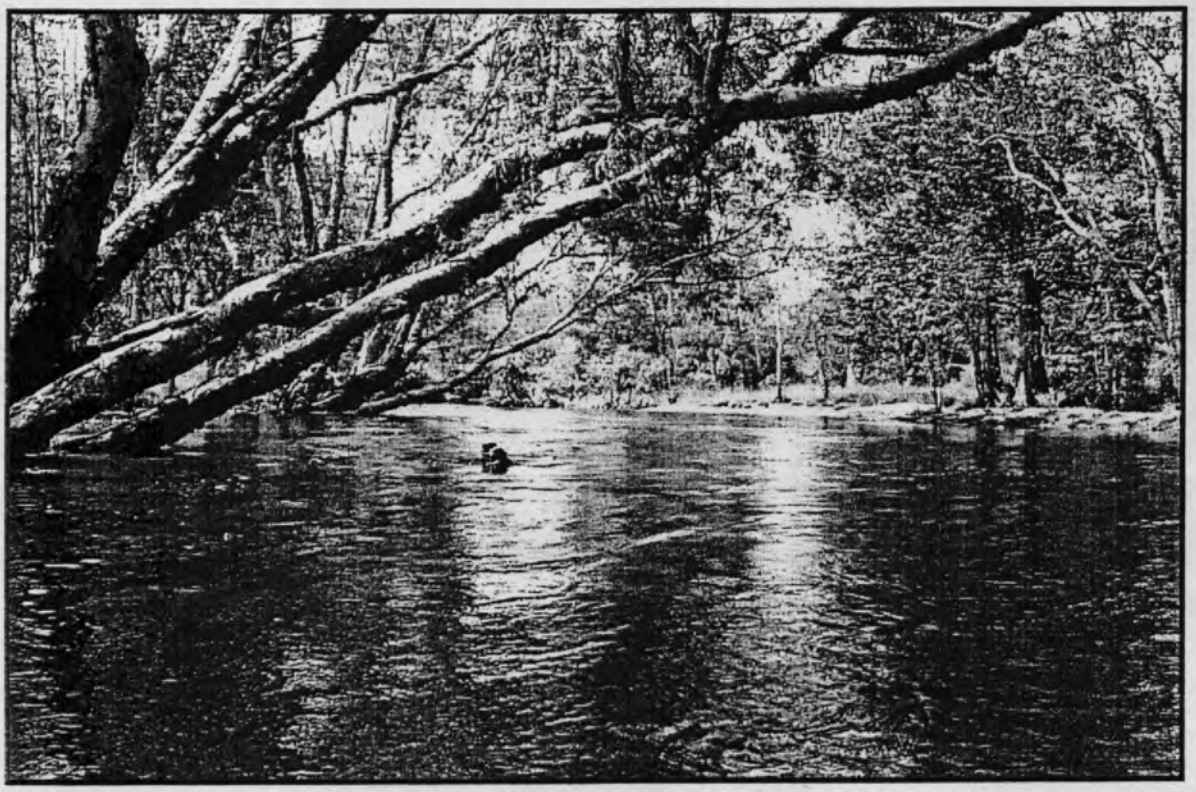


Figure 2.2: Photograph of Magela Creek Downstream of Georgetown Billabong, January 1999

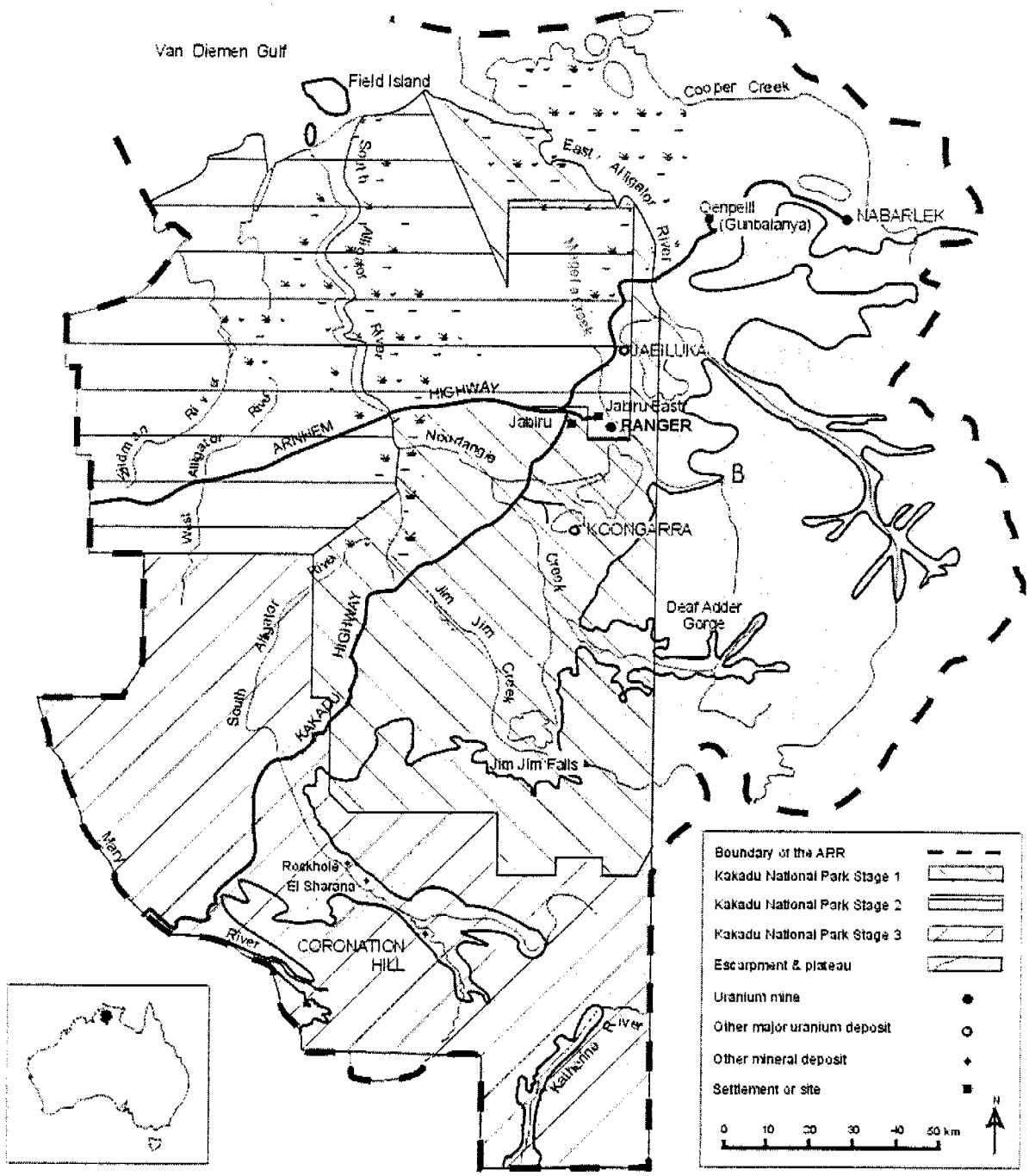


Figure 2.3: Map of Alligator Rivers Region and Magela Creek
A: Georgetown Billabong (275000E; 8597000N)
B: Bowerbird Billabong (283829E; 8592737N)

Table 2.1: Summary of Georgetown and Bowerbird Billabong water chemistry over two consecutive years

Metal	Georgetown (μg/L)		Bowerbird (μg/L)	
	24/5/99	18/6/00	23/8/99	16/7/00
	(Semaan, 1999)	(This Study)	(Semaan, 1999)	(This Study)
Al	NR	9.91	17	NR
Ca	600	200	200	NR
Cd	NR	0.02	<0.02	0.07
Co	NR	0.06	0.07	NR
Cr	NR	0.2	0.20	NR
Cu	<2	1.05	1.20	NR
Fe	NR	40	110	NR
Mg	900	0.80	100	0.80
Mn	3.5	1.78	2.70	0.86
Ni	NR	0.52	0.30	NR
Pb	<1	0.21	0.06	NR
Se	NR	<0.1	<0.2	NR
Zn	<2	1.3	NR	NR
U	<0.1	0.07	0.13	0.15
SO_4-S	200-400	300	300	300
pH	~6.35	6.60(0.16)	~6.40	6.46 (0.11)
Cond. (μS/cm)	~15.0	21.71 (3.27)	~20	18.42 (1.31)
D.O (%)	-	96.15 (6.39)	-	95.04 (5.87)

mL of filtered creek water, 2 x 10^5 cells/mL *Chlorella* sp. and 1µl/mL of fermented fish food with vitamins (FFV). Daily maintenance of the cultures involved transferring each flea into fresh water with fresh food (i.e. *Chlorella* sp. and FFV). A record of stock culture development and reproduction was kept and the occurrence of any deaths recorded. Whilst changing water and food, trays were removed from the incubator and placed on warming trays set to 31 °C. Cultures were restarted with second brood neonates usually occurring every 3-4 days.

2.4 Production of *M. macleayi* for the feeding bioassay (initial protocol)

Parents of test animals were raised according to reproduction test protocols (Hyne *et al.*, 1996). Test animals were maintained according to the procedure developed by Orchard (1999), as follows. 200 second brood neonates, released within six hours of each other, were isolated into a covered bowl containing 1.5 L of test dilution water, 2 x 10^5 cells/mL *Chlorella* sp. and 1µmL./mL FFV. The bowl was placed in an incubator at 27 °C with a light regime of 12 h light: 12 h dark. Water, *Chlorella* and FFV were changed daily until the animals had released their first brood. Animals were ready for the start of the test following the release of their first brood.

2.5 Algae culture and harvest

Starter cultures, algal slopes, mass culturing and harvesting of *Chlorella* spp. required monthly preparation of MBL growth media (Appendix 8). Both starter and mass cultures were maintained over the testing period. Seven to ten days before commencing a mass culture, 1 mL of the older starter culture was transferred into a 250 mL Erlenmeyer flask contiaining 100 mL of half-strength MBL media. Mass cultures were innoculated when starter cultures were in an exponential growth phase. Approximately two litres of MBL were prepared for each mass culture at a pH of between 7.1 and 7.3. The flask was plugged with a non-absorbent cotton wool stopper which acted as an air filter. The media

was autoclaved for 40 minutes and completed upon cooling (Appendix 8). The media was let stand for at least one day before use. Before innoculation of the mass culture with the starter culture, a cell count was conducted on the new starter culture. The volume of starter culture required to create a cell density of 10^4 cells/mL was calculated and this volume was then aseptically transferred to the mass culture media. Flasks were transferred to a 27 ± 1 °C incubator on an oscillating digital shaker for 10-14 days or until cells reach a density of 2 x 10^6 cells/mL before harvesting. Harvest of the mass culture involved centrifuging 12 x 80 mL plastic tubes containing 60 mL of the culture at 2800 rpm (2000 g) for 20 minutes at a temperature of 15 °C. Suction was used to remove the supernatant leaving approximately 5 mL of water in the base of each tube for re-suspension of the pellet. The pellets were re-suspended and combined. Empty tubes were rinsed by adding 20-30 mL of sterile dilution water, capped and vigorously shaken. The same aliquot was transferred from one tube to another and the final tube was re-spun. This procedure was then repeated until all algal cells were concentrated into one tube. The cells were washed twice with sterile dilution water to remove any trace metals from the media, these were centrifuged and the supernatant removed. The pellet was re-suspended in 50 mL of sterile dilution water. The algal density of the suspension was calculated to determine the amount of algae required in 30 mL to achieve a cell density of 2 x 10^5 cells/mL. 1 mL of the suspension was transferred to a volumetric flask and made up to 100 mL with sterile dilution water. The flask was then inverted five times and counts were taken using a haemocytometer (Appendix 7). Two litres of culture produced approximately 8.2 x 10^9 algal cells. The suspension was divided into 1mL aliquots and stored in the dark at 4 °C.

2.6 Production of fermented fish food supplement (FFV)

FFV was produced by blending 5.0 g of cichlid food, 0.25 g of powdered alfalfa and 250 mL of Milli Q water until homogenised. The mixture was then covered with a fine mesh and aerated for three days at an ambient water temperature of 25-28 °C. After settling in a

beaker for one hour the supernatant was decanted and 1 mL aliquots transferred into plastic vials. These vials were stored in a freezer and thawed for testing purposes or stock cultures.

2.7 Preparation of stock solutions

Metal salts (e.g. copper sulphate, cadmium chloride and uranyl sulphate) were dissolved in Milli Q water to attain a concentration of 10 mg/L. Solutions were refrigerated in plastic screw-capped bottles until required for use. If solutions were known to degrade they were prepared on the morning of the test. Prior to the commencement of the test, a 1/20 dilution of the stock solutions was made using filtered test dilution water to produce a 500 μg/L solution. The 500 μg/L solution was diluted to attain the required treatment concentrations.

2.8 Chemical analysis

Stock and treatment solutions were prepared as per the developed protocol (Section 2.7). A 50 mL sample was taken from each treatment solution at the start of each reproduction test and feeding test and transferred to polyethylene bottles that had been acid washed with nitric acid and rinsed with Milli Q water five times. Each sample was acidified with HNO_3 to 0.1 %. Chemical analysis for the final day of the feeding test was discontinued after noting little change in metal concentrations over the test durations (Orchard, 1999). ICPMS analysis of samples was performed by ChemNorth (Darwin) for metals and other inorgainics, and pesticides and surfactants were analysed by Algal (Sydney). Concentrations of analysed substances are listed in Appendices 4 and 5 .

2.9 Statistical analysis methods (general)

Statistical endpoints for most tests were determined using the data analysis package, Toxcalc™. This package enables all required toxicity indicies to be calculated using

parametric and non-parametric statistical methods. Analysis of the data was performed using a range of methods including Steels Many-One Rank Test (non-parametric) and Dunnett's Multiple Comparison Test (parametric). LOEC and NOEC values were obtained using hypothesis testing and point estimates were calculated using Maximum Likelihood Probit Analysis for Acute Immobilisation tests and Linear Interpolation for Reproduction and Feeding tests. Point estimates were compared using Standard Error of Difference (Sprague and Fogels, 1976). Where a toxicant was not used, data were analysed in Minitab, using 1 and 2 way ANOVA's to calculate significant differences and *post-hoc* analysis performed using Tukeys Pairwise Analysis. Data was also transferred to the graphics and data analysis package (Origin) which enabled visual assessment of the data. Prior to use in Origin data was manipulated in Microsoft Excel to derive averages, and 95 % confidence intervals.

2.10 Standard testing protocols

2.10.1 Feeding test (initial)

This protocol was developed by Orchard (1999).

Stock solutions were prepared in two litre volumetric flasks, according to the stock solution protocol (Section 2.8), and transferred to plastic screw top bottles for refrigeration. Appropriate dilutions of the stock solution were made on the morning of the test to attain the required treatment concentrations. 50 mL of each treatment was dispensed into eight polyethylene nalgene bottles and acidified to 0.1 % HNO_3 for chemical analysis. 500 mL of each treatment solution were measured and transferred into labelled glass screw top containers. *Chlorella sp.* were added at an algal density of 2 x 10^5 cells/mL. 49 (45 mL) plastic vials in seven treatment rows labelled A-G were arranged. Each treatment was shaken and 30 mL dispensed into corresponding vials (*e.g.* Treatment A into vials A1-A7). Before test commencement, vials were covered and stored in an incubator at 27 °C awaiting addition of test animals. Plastic vials (100 mL),

containing 60 mL of each treatment were used to measure pH, conductivity and dissolved oxygen prior to test commencement (T_0). Filtered dilution water (30mL without algae) was dispensed into two additional vials labelled CB1 and CB2 (Coulter blank 1 and 2).

Immediately following the release of their first brood, four adults were transferred from the culture vessel into each of the first five vials of treatments A-G working from lowest concentration to highest concentration. The final two vials in each treatment row were considered as treatment blanks and did not contain animals. The 49 treatment vials and the two coulter counter blanks (CB1 And CB2) were placed on perspex trays in random order. Trays were also placed in random order, in a dark incubator at 27 °C (Figure 2.4). Algal counts using Coulter Counter protocols (Appendix 10) were taken for 30 mL of each treatment (containing 2 x 10^5 cells/mL *Chlorella* sp.) and record as T_0. This process was repeated for the Coulter blank.

Vials were removed from the incubator after 20 hours incubation. All animals were removed and their status (dead/alive) and the presence of neonates recorded. If less than 3 adults were alive per vial the replicate was considered invalid for algal cell counts. Cell counts for each vial were taken using the standard Coulter counter protocol (Appendix 10). If the mean count for the blank differed by more that 15 % from the T_0 mean count for that treatment another count was taken with a second sample. If still outside 15 %, a set of counts were taken from the second blank for that treatment (i.e. A7). This step was repeated for the first blanks of the other treatments (i.e. B6-G6) and counts recorded as T_{20}. Counts were taken for each of the treatment vials (i.e. A1-5 through to G1-5).The remaining water from treatment vials (including the test dilution water vials) was poured into separate 100 mL vials and pH, conductivity, and dissolved oxygen values were measured (T_{20}).

Calaulation of feeding rate

Coulter counts were obtained for one replicate of each treatment and a Coulter blank (dilution water) at T_0, and five replicates, two treatment blanks and one Coulter blank for

Figure 2.4: Photograph of Initial *M. macleayi* Feeding Test (Orchard, 1999)

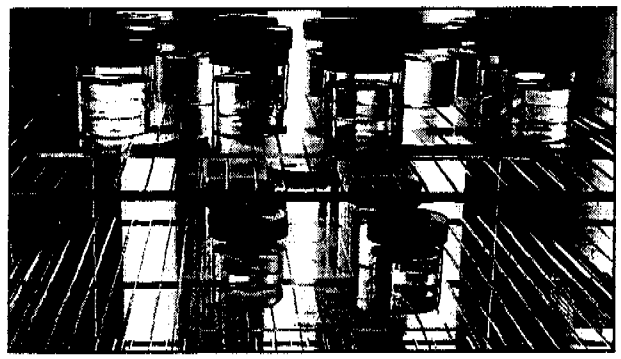


Figure 2.5: Photograph of *M. macleayi* Acute Immobilisation Test

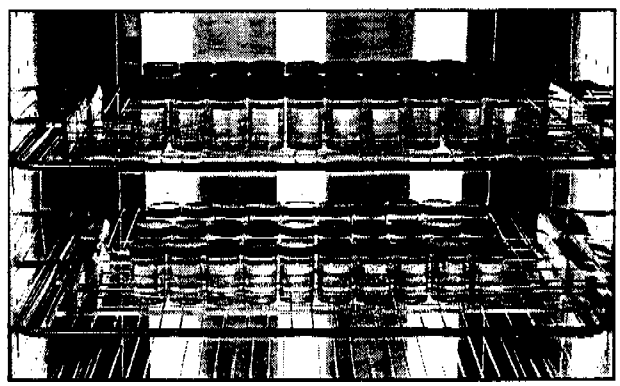


Figure 2.6: Photograph of M. macleayi 3-Brood Reproduction Test

each treatment at T_{20}. Five counts were averaged for each replicate, the Coulter blank subtracted and then multiplied by the dilution factor (i.e. x40 for a 1:4 and x20 for a 1:2 dilution). The T_0 and T_{20} treatment blank counts were averaged for each treatment to obtain initial counts in cells/mL.

Feeding rate was calculated for each replicate using a simplified version of Gauld's equation (Gauld, 1951).

$$F = \frac{V.(Ci - Cf)}{n.t}$$

F = Feeding rate in cells/animal/h

Ci = Initial cell concentration (Average of T_0 and T_{20} treatment blank counts)

Cf = Final cell concentration (T_{20} replicate counts)

V = Test volume in mL

n = No. of animals

t= Test duration

Acceptability of test data

The data set of the test was considered to be valid if:

The incubator temperature remains within 27 ± 1 °C;

Controls have at least 3 surviving adults per replicate;

Changes in cell counts between T_0 and T_{20} are consistent for all treatments

Data sets for each group were considered acceptable if:

Three out of five replicates are valid

T_{20} pH is within 0.5 units of the T_0 pH

Dissolved oxygen is >70 % of the air saturation at a temperature of 27 °C

2.10.2 48h Cladoceran Acute Immobilisation/Lethality test

M. macleayi neonates (<6h) were exposed to a range of effluent dilutions for 48 h. Screw-capped vials (200 mL) were used, each contained 150 mL of test solution and 10 neonates. Each cap contained two, 5 mm ventilation holes. Seven treatments were usually used for each test, with each treatment containing two or three replicates (as specified). Vials were then placed in random order, in an incubator at 27 ± 1 °C with a photoperiod of 12 hours light to 12 hours dark (Figure 2.5). Test solutions were not renewed and the number of surviving *M. macleayi* was recorded upon completion of the test (i.e. after 48 h). Tests were considered to be invalid if control mortality exceeded 20 %. Dissolved oxygen, pH, and electrical conductivity were measured at T_0 and T_{48}.

2.10.3 Cladoceran (*M. macleayi*) 3-Brood reproduction test

Female *M. macleayi* neonates (<6 h) were exposed to a range of Cadmium concentrations and effluent dilutions. Treatments were prepared with ten replicate 45 mL vials per treatment. Each vial contained 30 mL of treatment solution, *Chlorella* sp. at a cell density of 2 x 10^5 cells/mL and FFV (1μL/mL) and was capped with a lid containing two, 2 mm ventilation holes. One neonate was transferred to each vial and all vials were randomised and placed in a constant temperature incubator (27 ± 1°C) with a photoperiod of 12 h light and 12 h dark. Test solutions and food were renewed daily upon observation of brood numbers. Numbers of offspring were recorded daily including juvenile mortality until the completion of the test (release of third brood offspring from control animals). Broods were summed for each adult Cladoceran. If control mortality exceeded 20 % or total offspring was less than 30 per adult the test was considered to be invalid. Electrical conductivity, dissolved oxygen and pH were recorded for all fresh (T_0) and old (T_{24}) treatment solutions (Appendix 2). Test setup for *M. macleayi* 3-brood reproduction test can be seen in Figure 2.6.

Chapter 3: Feeding Test Validation

3.1 Introduction

Feeding responses, as behavioural endpoints, have been acknowledged over time by many authors for their sensitivity and reduced test duration (Juchelka and Snell, 1995; Bitton *et al.* 1996). The use of feeding inhibition as a behavioral endpoint in tests using *M. macleayi* was first investigated by Orchard (1999). Development of initial protocols were not only aimed at maintaining a high degree of ecological relevance to the region but also to accommodate similar aspects of other Cladoceran bioassay protocols, so that direct comparisons could be made between feeding and reproductive responses. Results from experiments by Orchard (1999) provided reasonable evidence that the test could estimate toxicity to a certain degree, with the test protocol being sensitive to cadmium but less so to copper and uranium.

The initial feeding test protocol had not been reproduced since initial development in 1998. Small changes in creek water and stock cultures may have a significant impact on test results over time. Therefore, prior to further development of the feeding test, it was necessary to reproduce the initial protocol to ensure that algal depression, due to feeding by *M. macleayi*, was detectable and that sensitivity to cadmium was comparable to results obtained by Orchard (1999). It was also necessary to compare Coulter counts with microscope counts to ensure that the Coulter counts are a "true" reflection of the algal concentration in the sample. The following null hypotheses were assessed in this chapter:

1. *There is no significant difference between algal cell counts using a Coulter counter or microscope haemocytometer.*
2. *M. macleayi feeding rates and algal depressions are significantly different from those obtained by Orchard (1999).*
3. *Cadmium has no effect on feeding or reproductive responses of M. macleayi*

3.2 Methods

3.2.1 Coulter counter validation

Microscope and Coulter counts were compared at T_0 and T_{20} for one treatment blank (A6), one Coulter blank (CB) and five treatment replicates (A1-A5). Microscope counts were obtained using a haemocytometer (Appendix 7) and Coulter counts were conducted following the Coulter counter protocol (Appendix 10). Treatments were prepared using filtered creek water, which had been filtered through filter paper with a pore size of 2.5 µm. The treatment blank contained 30 mL of filtered creek water and 2×10^5 cells/mL *Chlorella* sp. The Coulter blank contained filtered creek water only while the five treatment replicates contained 30 mL of filtered creek water, 2×10^5 cells/mL *Chlorella* sp. and 4 adult *M. macleayi,* added after the T_0 count. The test followed the initial protocol (Section 2.10.1). Coulter counts were diluted at a ratio of 1:4 before counting, however microscope counts required no dilution. Algal concentration and feeding rate were calculated for each vial at T_0 and T_{20}. (Note: the Coulter dilution factor was accounted for in this calculation). One-way ANOVA and Tukey's *post-hoc* analyses ($p<0.05$) were used to determine significant differences between the Coulter and microscope methods.

3.2.2 Investigation of feeding rates

Feeding rates were investigated, without a toxicant, using the initial feeding test protocol (Section 2.10.1). Five replicates were prepared, each containing 30 mL of filtered creek water, four adult animals per vial and an algal density of 2×10^5 cells/mL. Two further replicates contained only 30 mL of filtered creek water and an algal concentration of 2×10^5 cells/mL (i.e. no animals), while the Coulter counter blank (used to determine background algal counts in the creek water) contained filtered creek water only. The test was conducted for 20 hours in the dark at 27 ± 1 °C.

3.2.3 Investigation of cadmium toxicity

Cadmium toxicity was assessed using concurrent feeding and reproductive bioassays. The feeding test followed the initial protocol (Section 2.10.1) and reproduction was assessed using the standard 3-brood protocol (Section 2.10.3). Both tests were undertaken using filtered creek water collected from Georgetown Billabong. Seven treatments were prepared and analysed for actual cadmium concentrations. Due to an error in chemical analysis for one concentration, the measured cadmium level was determined using linear regression of nominal and measured cadmium levels for the other treatments.
LOEC, NOEC, EC_5 and EC_{20} estimates were derived using Toxcalc®. Point estimates were compared using Standard Error of Difference (SED) (Sprague and Fogels, 1976).

3.3 Results

3.3.1 Coulter counter validation

Initial counts (T_0) were not significantly different between the microscope and the Coulter, with mean counts (SD) of 1.25 (0.02) $\times$ 10^5 cells/mL and 1.91 (0.23) $\times$ 10^5 cells/mL, respectively. However, microscope counts and Coulter counts were significantly different at T_{20} ($p = 0.006$) with mean values (SD) of 0.96 (0.53) $\times$ 10^5 cells/mL and 1.93 (0.43) $\times$ 10^5 cells/mL, respectively.

3.3.2 Investigation of feeding rates

Mean feeding rates (SD) and algal depressions are shown in Table 3.1. A mean (SD) feeding rate of 3.30 (0.14) $\times$ 10^4 cells/animal/h and an algal depression of 36.6% (1.52) were obtained.

3.3.3 Investigation of cadmium toxicity

A response to cadmium was observed in both the reproduction and feeding tests (Figure 3.1). Point estimates, LOEC and NOEC values for this study and from Orchard (1999) are displayed in Table 3.2. Figure 3.2 compares feeding responses from this study and Orchard (1999).

Table 3.1: Feeding rates and algal depressions (SD) from this study and Orchard (1999)

Replicate	Feeding Rate ($x10^4$ cells/animal/h)		Algal Depression (%)	
	This Study	Orchard, 1999	This Study	Orchard, 1999
1	3.24	3.78	36	56
2	3.54	4.21	39	63
3	3.13	4.11	35	61
4	3.24	3.77	36	56
5	3.35	4.05	37	60
Mean (SD)	3.30 (0.14)	3.98 (0.19)	36.6 (1.52)	59.2 (3.11)

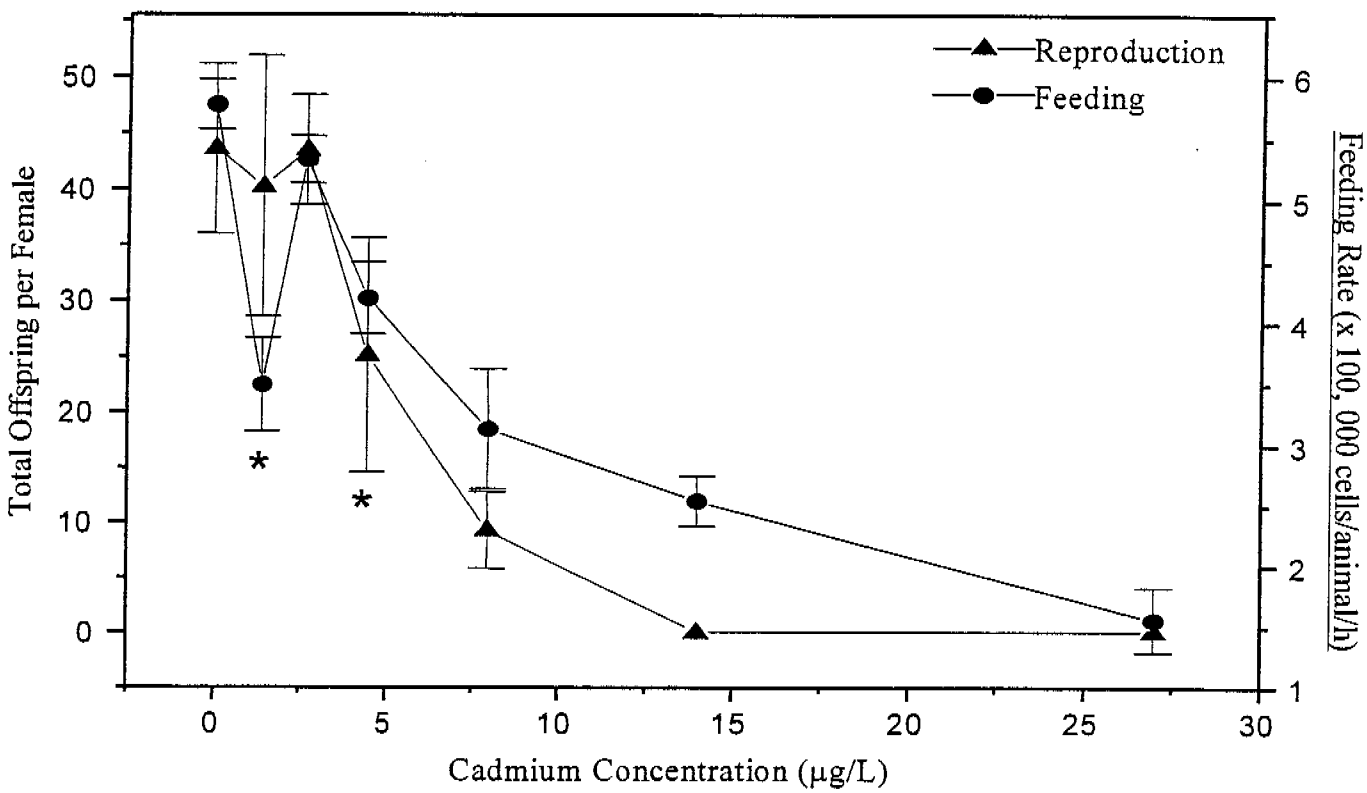


Figure 3.1: Effects of cadmium on the feeding rate (n=5) and reproduction (n=10) of *M. macleayi*: Results expressed as mean values (95%CI)
*LOEC values for each test

Table 3.2: Reproduction and feeding point estimates (95% CI) for this study and Orchard (1999)

	Present Study			Orchard (1999)	
	Test (1)	Test (2)	Test (3)	Test (1)	Test (2)
	Feeding	Feeding	Reproduction	Feeding	Reproduction
EC_5	0.3 [a]	0.55 [b,c,d]	2.72 [b]	0.80 [c]	1.30 [d]
	(0.24-0.41)	(0.03-3.93)	(0.33-3.02)	(0.0-1.0)	(0.9-1.9)
EC_{20}	1.20 [a]	1.82 [a]	3.41 [b]	5.90 [a]	1.8 [a]
	(0.97-3.81)	(0.30-4.38)	(1.34-4.48)	(0.00-7.50)	(1.4-3.2)
LOEC	1.41	4.50	4.44	1.8	3.1
NOEC	<1.41	2.68	2.68	1.2	1.7

N.B. Point estimate comparisons within rows .
Values with the same alphabetical superscript are not significantly different (P>0.05)

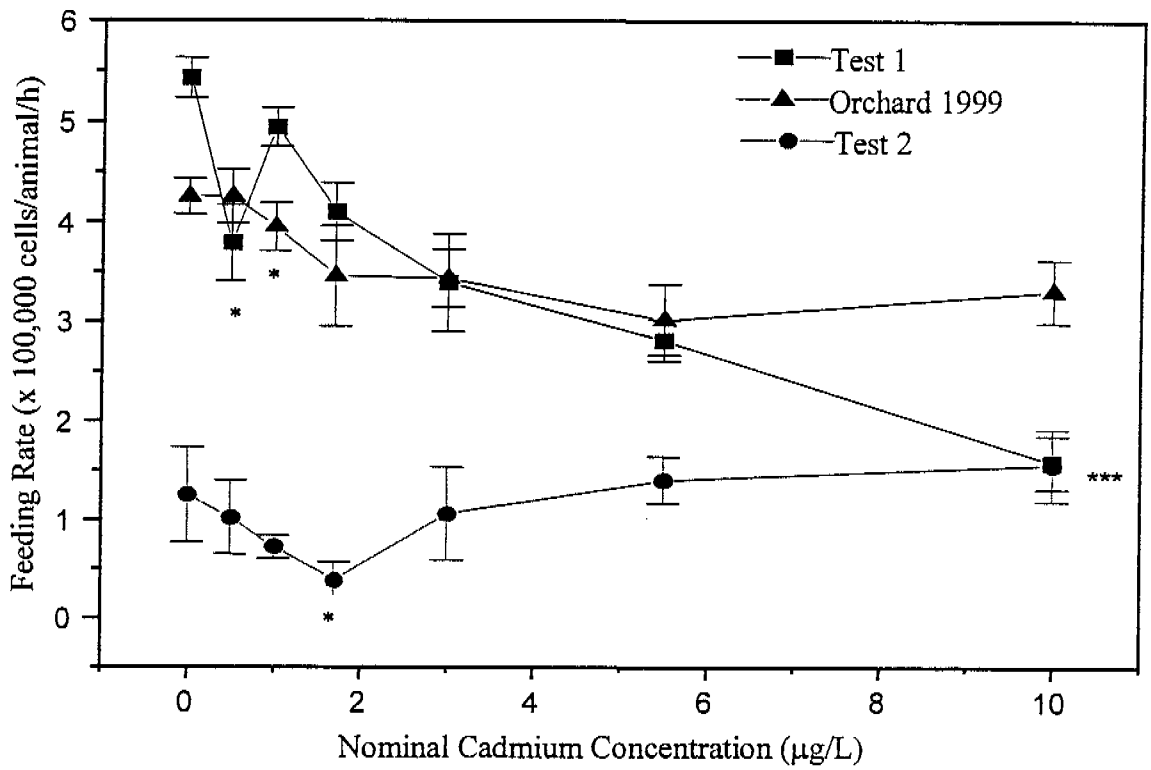


Figure 3.2: Feeding response of *M. macleayi* to cadmium in this study and Orchard (1999): Results expressed as mean values (95%CI)

*** False feeding rate increase due to changes in treatment blanks from day 1 to day 2 (Section 4.4.1)

* LOEC for each test

3.4 Discussion

3.4.1 Coulter counter validation

Initial test procedures involved the use of an electronic particle counter (Coulter Multisizer II) to determine ambient cell concentrations, instead of manual cell calculation. Although the initial protocol used the Coulter counter for cell enumeration, the accuracy of the machine had not been determined for the feeding test. Electronic cell enumeration was employed primarily to reduce the labour-intensive and often imprecise nature of manual cell counts using a haemocytometer (Stauber *et al.*, 1994). The Coulter counter requires algal cells to be suspended in an electrolyte solution (isoton), these are then passed through an electric current across a small aperture. As each cell crosses the current there is a voltage drop or pulse. Each pulse is proportional to the size of each cell, allowing the machine to calculate the distribution of cell sizes and numbers.

A common instrument fault is counting two cells as one. This leads to an underestimation of cell density, especially at high cell counts (>70 000 cells). To correct for this, the instrument includes a coincidence correction function. Good correlation between manual and Coulter counts have previously been observed, providing cell counts are between 4,500 and 70,000 and coincidence correction is below 35 % (Franklin, 1998). Despite this recommendation, the present study also found a good correlation between counts for background particles in the Coulter blank (CB) even though counts averaged ~1700.

In general, electronic cell counts matched the microscope counts at T_0. However, at T_{20}, electronic cell counts did not correlate with microscope counts for replicates containing animals. The Coulter counter had previously been calibrated in the development of an algal growth test (Franklin, 1998). During this process a similar method was followed to determine the accuracy of the machine and an excellent correlation between microscope and Coulter counts was found (r^2=0.98). Therefore the discrepancy, in this test, was attributed to animal debris and waste products counted as alga particles. Results from further testing gave similar results to this initial experiment suggesting that the range of

the Coulter counter needed to be adjusted in order to eliminate these particles. Results from these experiments are located in sections 4.1 and 4.2 and will be discussed later.

3.4.2 Investigation of feeding rates

One of the main considerations for each feeding test is to achieve an appropriate control feeding rate; an absence of this would nullify the test. Although individual feeding rates were variable for this study, mean feeding rates correlated closely with control rates attained by Orchard (1999). This variation is likely to be linked with problems associated with Coulter and microscope counts (Section 3.1). Small deviations in control feeding rates between experiments were also experienced, however, at this stage the main focal point was the detection of an appropriate feeding rate. In this case, all results demonstrated appropriate feeding responses and therefore supported the continued use of feeding rate as an appropriate endpoint for the test.

Another consideration was the degree of algal depression from initial concentrations after 20 hours of feeding. Ultimately, this should be high enough to obtain a dose-response relationship, but low enough to avoid food limitation. A suggested optimum algal depression was considered to be between 20-30 % of the initial cell density. However, slight deviations from these levels (e.g. 61 %) were recorded by Orchard (1999).

3.4.3 Investigation of cadmium toxicity

All experiments exhibited a high degree of sensitivity to cadmium, allowing comparisons between reproductive and feeding responses to be made. EC_{20} estimates correlated closely with those obtained by Orchard (1999) and therefore demonstrated that cadmium sensitivity had not changed significantly over time. EC_5 estimates were variable, however, this was possibly due to the unreliable nature of point estimates less than 5%, and is not necessarily a reflection on test results. The use of LOEC and NOEC values in these tests was undesirable due to consistent underestimation of toxicity. For these reasons point estimates greater than 5% were used to compare data in all following tests.

In past years traditional LOEC and NOEC values, or hypothesis testing has had considerable use in toxicity assessment. However, these methods have been subjected to criticism due to the nature of their calculation. One downfall is that values are restricted to a test concentration which may not provide an accurate measure of toxicity depending on experimental design. In addition, the NOEC depends greatly on the power of the test and because many ecotoxicological tests often exhibit low power it is often not reliable to determine the concentration at which there is no observed effect. To account for this, many alternatives have been suggested including the use of point estimates (Camilleri *et al.*, 1998). Point estimate methods derive concentrations estimated to affect a certain percentage of test organisms. Point estimates can represent a more accurate estimation of toxicity as they incorporate all information gathered from the test in the calculation of the values.

Major evidence for the use of point estimates includes the following (Moore and Caux, 1997)

a) Values are not restricted to specific test concentrations and therefore more biologically relevant thresholds can be determined.
b) Underestimation of toxicity is eliminated from the use of small sample sizes, improper spacing of treatment doses and large inter-treatment variance.
c) Levels are derived using all test data and therefore a more accurate estimation of effect can be determined.
d) Type II error, commonly experienced using hypothesis testing, and is eliminated.

Feeding rate "increased" with elevated levels of cadmium in feeding test 2 (Figure 3.2). Upon closer examination of these results, this response appeared to be an artifact of 'true' feeding rate due to changes in treatment blank counts over time. Currently, feeding rate is calculated by subtracting final cells/mL from the initial cells/mL with initial cell density being an average of T_0 and T_{20} treatment blank counts. Although this method corrects for

any small increases in the treatment blank over time it does not take into consideration any large changes as a result of Coulter counter changes from day to day. This factor can be corrected for by using the treatment blank values at T_{20} instead of an average of T_0 and T_{20} values, hence eliminating any daily variation with the Coulter counter. Alteration of the feeding rate formula will be discussed in section 4.

3.5 Conclusions

The validation of initial test protocols led to the following conclusions:

1. Coulter counts did not correlate with microscope counts at T_{20} as a result of debris in the sample being counted as "algae". Further investigation of this factor will aim to achieve a more accurate estimation of algae in the sample.

2. A suitable feeding rate per animal was achieved, supporting the use of feeding inhibition as a favorable endpoint.

3. Cadmium sensitivity was noted in all experiments with more sensitive responses obtained for this study than experiments using adult organisms..

Chapter 4: Development

4.1 Introduction

Preliminary results have supported the use of feeding rate as an appropriate endpoint for toxicity assessment for single metals and possibly waste waters. Recent validation (Chapter 3) of initial feeding test protocols, developed in 1999 (Section 2.10.1), highlighted a range of problems. In this section, further development of initial test protocols was undertaken with the following objectives in mind.

a) To simplify testing procedures by reducing test volume and algal cell density and investigating the use of sample storage by refrigeration.
b) Increase the sensitivity and simplify testing procedures by using *M. macleayi* neonates.
c) Increase the consistency and reliability of the test by simplifying the feeding rate formula and optimising counting techniques.
d) Investigate the sensitivity of the final feeding test protocol to cadmium.

Rationale for each section is mentioned briefly in the methods. The following null hypotheses were assessed:

1. *Substitution of T_{20} treatment blank counts as initial values does not effect feeding rate calculations.*
2. *Culture of more than one animal per vial has no effect on the survival, growth and reproduction of M. macleayi.*
3. *Reductions in test volume and animal numbers have no effect on feeding rates or algal depressions.*
4. *Feeding rates and algal depressions for M. macleayi neonates over 20 hours are not measurable.*
5. *Waste and debris from neonate M. macleayi have no effect on Coulter counts.*
6. *Alteration of the counting range has no effect on Coulter counts.*
7. *The use of a 1:4 Coulter dilution factor does not change ambient cell counts.*
8. *Refrigeration of samples has no effect on algal cell counts.*

9. *M. macleayi neonate sensitivity to cadmium is not significantly different from adults.*
10. *Reduction in cell density has no effect on feeding rate and algal depressions of neonate M. macleayi.*

4.2 Methods

4.2.1 Evaluation of the feeding rate formula

Algal changes over the 20 hour test duration were assessed using a control vial (treatment blank) containing 2×10^5 cells/mL *Chlorella* sp. and 30 mL of treatment water. Changes in Coulter counts, over the 20 hour test period, should be eliminated as "immobilised" *Chlorella* cells generally do not divide in a dark incubator. However, experiments by Orchard (1999) and in this study have identified changes in T_0 and T_{20} treatment blank counts (Section 3.3.1). In the occurrence of such changes, the use of initial T_0 counts as initial values would ultimately lead to "false" representations of feeding rate (Figure 4.1). Therefore adjustments to the initial feeding test formula were made to eliminate this problem. Orchard (1999) used an average of counts, at T_0 and T_{20}, as initial values to calculate feeding rate (Orchard, 1999). In this study, treatment blank counts at T_{20} were used instead of initial values, in an effort to eliminate day to day variation in Coulter counts, and produce a more accurate representation of feeding response. Feeding rates for two existing experiments, one using adult animals and the other neonates (Section 4.4), were calculated using T_0 treatment blank counts (Gauld's Formula; Gauld, 1951), an average of the T_0 and T_{20} treatment blank counts (Orchard, 1999) and T_{20} treatment blank counts. Statistical analysis could not be performed due to insufficient data points, however significant differences between values could be estimated graphically.

4.2.2 Effects of culturing more than one animal per vial

Animals used in the initial feeding test protocol were produced in a labour intensive bowl culture involving the transfer of approximately 200-second brood neonates into fresh water, food and FFV for up to 4 days. Even after completion of this step there was no guarantee that animals would produce broods concurrently, leading to difficulties with

test start procedures, arising from lengthy waiting periods and the constant monitoring of animals. To overcome this problem, the culture of feeding test animals in vials was investigated. Animals can be successfully maintained one per vial as stock cultures, however, it would have been time consuming and impractical to culture 200 animals in 200 separate vials. For this reason the effects of culturing more than one animal per vial was assessed. *M. macleayi* reproduction protocols were followed to assess growth, brood size and mortality (Section 2.10.3). Each vial, per treatment, contained 30 mL filtered creek water from Georgetown Billabong, 30 μg/mL FFV and 2×10^5 cells/mL *Chlorella*. Treatments consisted of 1, 2 and 3 animals per vial with ten replicates of each treatment.

4.2.3 Reduction of test volume

A reduction of treatment volume would not only eliminate the preparation of large treatment volumes, but also allow for a reduction in the number of test animals and possibly the use of neonates (Section 4.2.4). At this stage of development, maintenance of the suggested 20-30 % algal depression was of importance to avoid food limitation. However, a workable volume was also required if another sample needed to be taken and re-counted in the event of a Coulter counter error. The optimal number:volume ratio was determined using four treatments, each with six replicates (five replicates and a treatment blank). Treatments consisted of 4 adult animals in 30mL (A), 2 animals in 15 mL (B), 2 animals in 10mL (C) and 3 animals in 10 mL (D). The test was run as per the initial feeding test protocol (Section 2.10.1) in the absence of a toxicant. Feeding rate per animal and algal depression was determined and compared to the feeding rates of the initial number: volume combination (*i.e.* 4 adult *M. macleayi* in 30mL).

4.2.4 The use of neonates

Research on a range of test species has concluded that testing juvenile stages are generally more sensitive than mature forms. Hence the use of neonates can potentially increase the sensitivity of the test (Baird *et al.*, 1991). This, along with culturing difficulties (Section 4.2.2), has prompted the investigation of neonate *M. macleayi* (<6 h)

in this research. Whilst an algal depression of 20-30 $\pm$ 10 % was important, the main objective at this stage was the detection of an appropriate feeding rate while maintaining a practical test protocol. Neonate feeding rate, in the absence of a toxicant, was assessed on two occasions using six replicates of three, four, five and six neonates per 15 mL of filtered creek water. The test was run for 20 hours in the dark at 27 $\pm$ 1 ∘C. Results were then compared to the algal depressions and feeding rates for adult animals.

4.2.5 Coulter counter validation (neonates)

The Coulter counter records particle distributions and cannot distinguish between algae and waste, therefore any particle falling within the designated size range will be included in algal cell counts. Particle size for waste and food debris from adult animals has already been determined to have an effect on the correlation between Coulter and microscope counts. However, waste particles from neonates may be significantly smaller and therefore any discrepancies between Coulter and microscope counts needed to be assessed. T_0 and T_{20} counts were taken for a total of seven vials containing 30 mL of filtered dilution water (two treatments, each with three replicates and a Coulter blank). Treatment A contained 2×10^5 cells/mL *Chlorella* and Treatment B contained 2×10^5 cells/mL *Chlorella* with five neonates added after the T_0 count. The test was run in the absence of a toxicant for 20 hours in the dark in an incubator at 27 $\pm$ 1 ∘C. Microscope counts were taken using a haemocytometer (Appendix 7), and Coulter counts using standard protocol (Appendix 10). One-way ANOVA and Tukey's *post-hoc* analysis was used to analyse data.

4.2.6 Defining the Coulter range

The Coulter counter consists of a multichannel system covering a particle range of 0.4 μm to 1200 μm. "Full" range counts can be adjusted to count either 64, 128, or 256 channels, with additional counts then being performed over two successive screens, the "narrow" and the "window". By positioning the two on-screen cursors it is possible to narrow the counting range on each screen. Fine-tuning of this range should reduce the

possibility of waste particles counted as algae. Two Coulter blanks and three treatment replicates were prepared according to the initial feeding test protocol (Section 2.10.1), with each replicate containing 2×10^5 cells/mL of *Chlorella*, 15mL filtered creek water and 5 neonate *M. macleayi* (>6 h). Coulter blanks contained filtered creek water filtered through a pore size of 2.5 μm. Coulter counts were taken, once for each vial at T_0 and multiple times at T_{20} using a series of particle ranges. Microscope counts were also taken for comparative purposes for each vial using a haemocytometer (Appendix 7). Comparison between microscope counts and Coulter counts highlighted the Coulter range that provided the best estimate of algal cell concentration for all samples. This range was re-validated using five treatment replicates (A1-A5), one treatment control (A6) and one Coulter blank (CB). All vials were counted at T_0 and T_{20} using the defined Coulter range of ~2.4-3.9. Microscope counts were also taken for all vials using a haemocytometer (Appendix 7). One-way ANOVA and Tukey's *post-hoc* analysis was used to determine significant differences between the Coulter and microscope methods.

4.2.7 Investigation of dilution rate

A Coulter count of approximately 5000 cells was required for accuracy. The upper limit was also restricted, and samples with a high algal content required the application of a specific dilution factor before counting. A feeding test was conducted using five *M. macleayi* neonates in 15 mL of test dilution water. After 20 hours the animals were removed and samples prepared for counting. Both 1:4 and 1:2 dilutions of the sample were prepared with isoton and counted for each of the 10 vials at T_0 and T_{20} using standard Coulter counter protocol (Appendix 10). One-way ANOVA and Tukey's *post-hoc* analyses were performed to determine significant differences between the 1:2 and 1:4 dilutions.

4.2.8 Effects of refrigerating samples

Five feeding test replicates containing five neonates, in 15 mL of test solution and *Chlorella* at a density of 2×10^5 cells, were prepared and placed in a dark incubator at 27

$\pm$ 1◦C for 20 hours. Coulter counts were taken at T_0, T_{20} and T_{40} (after 20 hours of refrigeration). Providing no effects were observed for Coulter counts from $T_0 - T_{x\text{-}20}$, the provision for counting up to 20 hours after test completion could be made. One-way ANOVA and Tukey's *post-hoc* analyses were performed.

4.2.9 Investigation of cadmium toxicity (neonates)

Initial test protocols, using adult animals, demonstrated a high degree of cadmium sensitivity. However, following the recent change to neonates (Section 4.2.4) it was necessary to re-assess feeding test sensitivity. The feeding test was prepared following the initial test protocol (Section 2.10.1). Five neonate animals in 15 mL of test solution were used as opposed to four adult animals in 30 mL. Seven test concentrations were prepared and chemically analysed for actual cadmium concentrations (Appendix 4). Algal cell counts were taken at T_{20} and T_{40} after refrigeration. A concurrent reproduction test was conducted using the standard test protocols (Section 2.10.3). EC_{10}, EC_{20} and EC_{50} estimates were derived using Toxcalc®.

4.2.10 Effects of cell density on feeding rate

The recommended cell density for culturing *M. macleayi* is approximately 2×10^5 cells/mL. This density is currently used for the culture of stock at *eriss*. Whilst this concentration is required for stock cultures, dealing with one adult and up to 25 neonates for 24 hours, it is not necessary for this amount of algal saturation to occur in the feeding test which supports five neonates for 20 hours. Reducing algal concentration would also provide a more appropriate algal depression as neonates have substantially lower feeding rates than adult animals. A reduction in cell density from 2×10^5 to 1.5×10^5 cells/mL was investigated. The test used five neonates in 15 mL. Vials were incubated for 20 hours in a dark incubator at 27 $\pm$ 1 ◦C. One-way ANOVA and Tukey's *post-hoc* analyses were performed.

4.3 Results

4.3.1 Evaluation of the feeding rate formula

Although changes to Gauld's formula had no overall effect on the calculation of NOEC and LOEC values, a more accurate representation of dose-response for both adult and neonate feeding tests was obtained when T_{20} counts were substituted for initial counts in Gauld's formula (Figure 4.1).

4.3.2 Effects of culturing more than one animal per vial

Both health and reproductive quality was maintained in vials containing one animal, supporting all prior knowledge of *M. macleayi* culture techniques. Two animals per vial provided a similar response. However, an increase to three animals per vial had noticeable decreases in health, with these animals exhibiting a 30 % death rate. Reproductive quality was also compromised with mean (SD) third brood offspring reduced to 14 (5.07) per animal in comparison to 20 (1.18) and 19 (2.37) for one and two animals per vial, respectively.

4.3.3 Reduction of test volume

Feeding rates and algal depressions for adult *M. macleayi* at different number/volume combinations are outlined in Table 4.1. Mean feeding rate (95 % CI) for the vials containing two animals in 15 mL was 4.96 (4.54-5.38) $\times$ 10^4 cells/animal/h, in comparison to the control feeding rate of 4.34 (4.05-4.63) $\times$ 10^4 cells/animal/h (4 adults in 30 mL). Treatments containing 2 and 3 animals in 10mL were not suitable as feeding rates for these replicates did not correlate with controls, and algal depressions were unusually high (Table 4.1).

4.3.4 The use of neonates

Feeding rates and algal depressions for each number:volume combinations are displayed in Table 4.2. Five neonates in 15 mL proved to be the most appropriate concentration

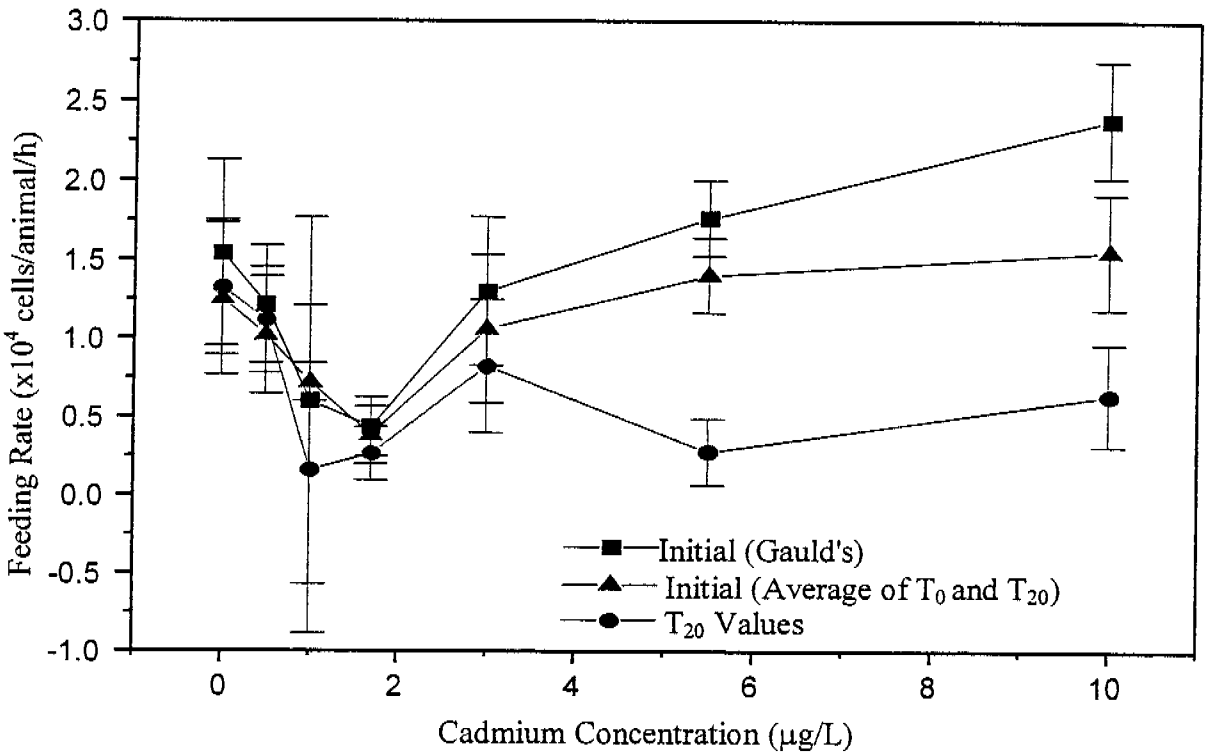


Figure 4.1: Adult *M. macleayi* feeding rates for increasing cadmium concentrations (feeding rate is calculated using different initial values)

Table 4.1: Mean feeding rates and algal depressions (95%CI) with varying number/volume combinations

Treatment	Feeding Rate ($x10^4$ cells/mL)	Algal Depression (%)
4/30mL	4.34 (4.05-4.63)	65 (60.52-69.48)
2/15mL	4.96 (4.54-5.38)	67 (61.24-72.76)
2/10mL	3.27 (3.04-3.50)	74 (69-79)
3/10mL	3.02 (2.79-3.25)	68 (62.53-73.47)

Table 4.2: Comparison of mean feeding rates and algal depressions (SD) for adult and neonate *M. macleayi*

	Treatment	Feeding Rate ($x10^4$ cells/animal/h)	Algal Depression (%)
Adults	4/30mL	3.30 (0.14)	36.6 (1.52)
Neonates	3/15mL	1.16 (0.94)	18.7 (14.7)
	4/15mL	1.17 (0.30)	28.4 (7.84)
	5/15mL	1.13 (0.22)	32.4 (5.21)
	6/15mL	1.14 (0.13)	40.6 (5.48)

with a feeding rate of 1.13 (0.22) × 10^4 cells/animal/h and a mean algal depression of 32.4 (5.21) % being obtained.

4.3.5 Coulter counter validation (neonates)

There was no significant difference between Coulter and microscope counts at T_{20} for samples with and without test animals ($p = 0.89$). At T_{20} mean Coulter counts (SD) for samples with and without test animals were 10.30 (0.76) and 16.99 (2.08) × 10^4 cells/mL, respectively. Corresponding microscope counts were 9.92 (2.27) and 19.08 (1.01) × 10^4 cells/mL. There was no significant difference between T_0 counts for the Coulter counter and the microscope with mean algal densities (SD) of 19.76 (0.67) and 22.00 (1.40) × 10^4 cells/mL recorded for the Coulter counter and microscope, respectively.

4.3.6 Defining the Coulter range

Coulter counts for samples 1-5 at various ranges are displayed in Table 4.3. Corresponding microscope counts did not match one particular range, however, the lower limit of counting was determined to be 2.278 μm and the upper limit 4.159 μm. Re-assessment of this range produced counts which correlated closely with microscope counts ($r^2 = 0.96$). Coulter blank counts were significantly different at T_{20} for microscope and Coulter counts, with algal densities of 3.42 and 0.75 × 10^4 cells/mL recorded for Coulter and microscope counts, respectively.

4.3.7 Investigation of dilution rate

There was no significant difference between 1:4 and 1:2 counts for all samples ($p=0.66$).

4.3.8 Effects of refrigerating samples

The mean algal count (SD) at T_{20} was 13.82 (1.57) × 10^4 cells/mL in comparison to 13.92 (1.56) × 10^4 cells/mL after 20 hours of refrigeration. There was no significant difference between refrigerated counts and T_{20} counts ($p = 0.912$).

Table 4.3: Coulter and microscope counts at various Coulter ranges (μm)

Range	Mean Coulter Count (cells)	Coulter count (Cells/mL x 10^4) Non blank-corrected	Microscope count x (10^4Cells/mL)
1.925-6.898	5867	23.47	-
2.721-4.159	4092	16.36	-
2.430-4.159	5031	20.12	-
			20.75
1.985-6.898	5434	21.73	-
2.644-3.732	3113	12.45	-
2.313-3.732	3455	13.82	-
			11.30
1.925-6.898	4174	16.70	-
2.547-3.868	2733	10.93	-
2.721-3.693	2900	11.60	-
1.925-6.898	4737	18.95	-
2.275-3.673	3808	15.23	-
			12.12
2.275-3.673	3832	15.33	-
1.925-6.898	4465	17.86	-
2.993-3.945	2422	9.69	-
2.760-3.945	2801	11.20	-
2.605-3.945	3200	12.80	-
			16.62
1.925-6.898	1182	4.73	-
2.547-3.868	557	2.22	-
2.721-3.693	419	1.67	-
2.993-3.945	864	3.46	-
2.760-3.945	823	3.29	-
2.605-3.945	933	3.73	-
			3.50

4.3.9 Investigation of cadmium toxicity (neonates)

Cadmium sensitivity was variable for neonates over the testing period as shown by point estimates in Table 4.4. Feeding responses are represented graphically in Figure 4.2.

4.3.10 Effects of cell density on feeding rate

There was no significant difference in feeding rates for replicates with varying cell densities (p=0.166). Mean feeding rates (SD) were recorded as 0.71 (0.27) $\times$ 10^4 cells/animal/h and 0.922 (0.15) $\times$ 10^4 cells/animal/h for the cell densities of 1.5 $\times$ 10^5 and 2.0 $\times$ 10^5 cells/mL, respectively. The reduction in algal concentration brought about an increased but more stable mean algal depression (SD) of 40.83 (3.06) % compared to 27.91 (34.83) % at 2.0 $\times$ 10^5 cells/mL. Decreases in food levels also brought about an increase in cadmium sensitivity (Table 4.4).

4.4 Discussion

4.4.1 Evaluation of the Feeding Rate Formula Accuracy

Treatment blanks are used as a control mechanism to monitor any significant changes in the algae from T_0 to T_{20}. This can sometimes be an indication of algal division or algal toxicity, even though non-dividing algal cells and dark conditions were used. Toxicant concentrations were also well below those causing noticeable algal toxicity. Throughout the course of test development (1998-2000) there have been large differences in treatment blank counts from T_0 to T_{20}. Four suggestions for this variation have been proposed;

(1) It is possible that the inorganic and organic composition of the creek water changes over time

(2) Changes in Coulter counter function from day-to-day

(3) Algal cell division in the dark

(4) High conductivity in treatment solutions causing fluid exchange across algal membranes, resulting in cell size changes. These cells may or may not be excluded by the Coulter counter range, hence potential variability in the counts.

Table 4.4: Cadmium point estimates (95% CI) for neonate feeding tests

Neonates	EC10	EC20	EC50
Test 1	4.39 (0.00-8.50) [a]	7.34 (0.00-21.58) [a]	25.40 (NA)
Test 2	1.61 (1.44-1.73) [b]	1.82 (1.65-2.07) [b]	2.45 (2.25-12.05) [a]
Test 3	2.19 (0.00-4.18) [b,a]	2.57 (0.00-4.37) [a,c]	3.70 (1.47-4.84) [a]

Comparisons are within columns where values with an alphabetical superscript in common are not significantly different ($p > 0.05$)
(NA) = 95% Fiducial Limits were not available

Initial cell density **(Test 1) = 2.0×10^5 cells/mL**

(Tests 2, 3 and 4) = 1.5×10^5 cells/mL

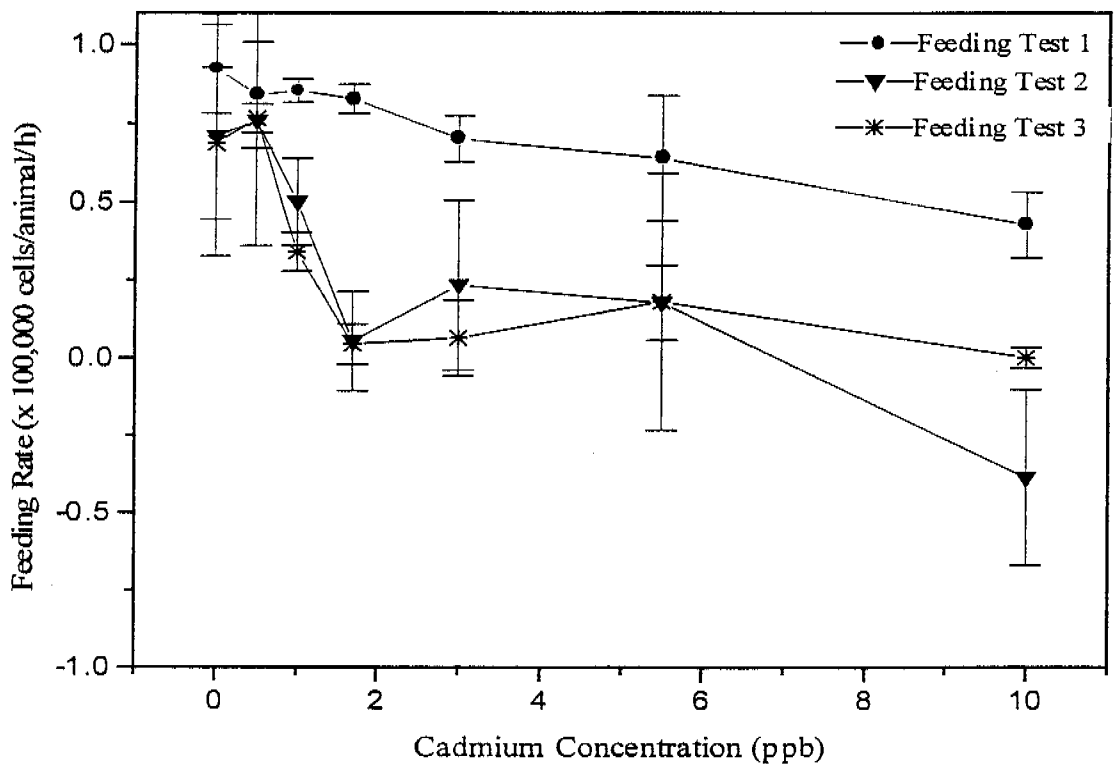


Figure 4.2: Effects of cadmium on *Moinodaphnia macleayi* neonates

In any case, it is important that this difference is recognised and factored into resultant calculations.

The feeding rate equation, developed by Gauld (1951), used initial T_0 counts to calculate feeding rate (Section 2.10.1). Attempting to reduce the effects of daily variation in treatment blank counts, Orchard (1999) modified this equation to replace initial counts with an average of the T_0 and T_{20} treatment blank counts. Although this counter-balanced some discrepancy between day-to-day counts, it also introduced the use of a inaccurate estimation of initial cell density, as this count could not be directly compared to counts on either day. A further formula change was then made, in this study. T_{20} counts were substituted for initial values in the calculation of feeding rate as it provided more accurate representations of dose-response. All counts used in the calculation of feeding rate were taken at T_{20} and therefore any changes in water parameters were eliminated and direct comparisons could be made for replicate vials of the same treatment. The use of T_{20} counts also eliminates any changes in counts due to disintegration of algae over the test duration, vacuum leaks and/or counting time changes experienced with Coulter counter function.

One disadvantage with using T_{20} counts is that algal toxicity cannot be readily detected. For this reason it is important that all T_0 and T_{20} treatment blank counts are compared and any discrepancies checked with microscope counts.

4.4.2 Effects of culturing more than one animal per vial

Bowl culture of animals for initial protocols has proven to be labour intensive and unreliable. If required, the use of vials for the culture of test organisms is viable at a density of two animals per vial. In vials containing three animals,food limitation and the accumulation of waste products was of concern, with the latter indicatedby significant increases in pH and conductivity. Increased stress, with three animals per vial, resulted in significant health and reproductive effects however, two animals per vial recorded no

reduction in health or brood size of the animals. Nevertheless culture of organisms in bowls or vials is still labour intensive and impedes otherwise simple protocols. It would be favourable to eliminate this step from procedures and therefore the use of second brood neonates (<6 h) as a replacement for adults was investigated and discussed below (Section 4.4.4).

4.4.3 Reduction of test volume

Previous assessment of volume/number combinations by Orchard (1999) concluded that 30 mL was the most appropriate volume where 4 adult animals were cultured, as 20 mL brought about concern for food limitation and stress. The primary objective of this experiment was to identify reduced volume/number combinations, providing direct comparisons to control feeding rates and algal depressions (i.e. 4 adults in 30 mL). In the current study, the most appropriate combination was determined to be two animals in 15 mL and therefore this volume was used for all further testing. Three animals in 10 mL also provided similar algal depressions to controls, however this volume proved inappropriate. At this volume water parameters could not be measured accurately, even when the water from all replicates was pooled. There was also no possibility of re-sampling in the event of a Coulter counter error. In this experiment algal depressions were high, although this may be associated with an increased feeding rate per animal. Increases in feeding rate may also be associated with photoperiod-related changes such as feeding and moulting cycles, or have a basis in the nutritional status of the animals. These factors are yet to be fully assessed.

4.4.4 The use of neonates

Toxicity tests over an organisms life cycle are considered to be the ultimate test for the establishment of "safe" concentrations of chemicals for release into the environment

(Rand and Petrocelli, 1985). During these tests it has been recognised that there are certain life stages that are consistently more sensitive than others. Therefore, these life stages have been used to develop faster and less costly methods of predicting chronic toxicity. In this research, *M. macleayi* neonates were used to not only increase the sensitivity of the feeding test, but also to eliminate labour intensive culturing methods.

Due to the relative size of neonates (~0.3 mm diameter at 4 hours) there was a significant reduction in feeding rate in comparison with adult animals (~1 mm at 49 hours) (Appendix 6). Sufficient animals were also required in each vial to enable a significant algal depression to be detected. Providing the optimum algal depression is 20-30 $\pm$ 10 %, five neonates in 15 mL was the most appropriate combination in both feeding tests, and therefore, this combination was used for subsequent tests. Although the use of four neonates in 15 mL provided similar results, this combination was not used due to the possibility of transfer error and highly variable results.

The chosen combination (5 neonates in 15 mL) requires the transfer of one more animal per vial than the initial protocol (4 adults in 30 mL). Since these animals are significantly smaller and more difficult to handle, there is a possibility of transferring one more or less into each vial. Though the possibility of transfer error has increased, feeding rates of each animal are smaller and therefore one additional animal will have little overall effect on feeding rate. This was demonstrated by the small difference between algal depressions and feeding rates for four and five neonates in 15mL (Table 4.2). Therefore, so long as there is at least four and no more than six animals per vial, this error can be justified more so than an error with adult animals.

It is also likely that neonates produce less waste and debris as a result of smaller filtering and feeding mechanisms. These particles may be mistaken as "algae" and subsequently included in Coulter counts. A Coulter counter validation experiment was undertaken for

neonates to detect and account for any differences in microscope and Coulter counts (Section 4.4.5).

4.4.5 Coulter counter validation (neonates)

There was no significant difference between Coulter and microscope counts. Closer examination of Coulter counts and microscope counts revealed that Coulter counts were consistently higher. Although this error was smaller than that encountered with adult animals, an accurate estimation of the algal cell density was still required for accurate test results. Therefore the counting range was adjusted on the Coulter counter to eliminate waste particles from the counts (Section 4.4.6).

4.4.6 Defining the Coulter range

No one counting range provided an overall correlation with all samples. The lower limit for counting was determined to be around 2.5 μm and the upper limit around 4.0 μm. However, a range needs to be chosen which will provide an accurate estimation of cell size for all samples, without the need to change the counting range between samples. A final range of 2.467-3.970 μm was decided to be appropriate for all samples. This range was twice validated to ensure there was good correlation between Coulter and microscope counts and no significant difference between counts was observed. This range was used for all further counting in the feeding test.

4.4.7 Investigation of Dilution Rate

Previous and current evaluation of the Coulter dilution factor has concluded that there was no significant difference between counts using a dilution rate of either 1:2 or 1:4 (Orchard, 1999). A 1:2 dilution was chosen for previous testing as it produced counts that were consistently above 5000 cells/mL. However, even using the 1:4 dilution, counts were commonly above 5000 cells/mL and a 1:4 dilution provided counts with a lower co-efficient of variation and within replicate variation. This enables more aliquots to be taken for sampling and re-counting which is favourable for the reduced volume of 15mL.

It is also consistent with other test protocols such as the algal cell density test using *Chlorella* sp. (Franklin, 1998). For these reasons the use of the 1:4 dilution was justified for all future feeding test counts.

4.4.8 Effects of refrigerating samples

In addition to maintaining a high degree of ecological relevance, it was also important to account for any test inconveniences such as start and finish times. *M. macleayi* release a new brood every 26 hours, and even though there is a six-hour window in which tests can be started, it is sometimes necessary to start tests outside normal working hours. This is inconvenient for commercial purposes that usually require tests to be undertaken during work hours. It is not possible to alter breeding times for *M. macleayi,* although it is possible to delay counting by preservation of feeding test vials by refrigeration. An important part of ecotoxicological testing is to maintain the same conditions throughout the test, so that any changes happen within test conditions. In general, refrigeration was used to preserve the sample at low temperatures (~4∘C) minimising the possibility of any changes in test solutions. A high correlation between T_{20} and refrigerated counts was found, allowing for test samples to be refrigerated for up to 20 hours before counting if required.

2.3.9 Investigation of cadmium toxicity (neonates)

M. macleayi neonates are notably susceptible to cadmium and have exhibited similar if not increased sensitivity in comparison to adults in this study. Increased sensitivity of neonates may be due to factors including more frequent moulting during earlier life stages. Moulting is a natural process for all crustaceans and any interruption to this cycle may result in an increased sensitivity to toxicants. In addition, neonates provide culturing advantages and simplification of start procedures. Therefore *M. macleayi* neonates were justified for use in all further testing.

4.4.10 Effects of cell density on feeding rate

Ambient food levels are known to influence growth, metabolism and reproduction in many cladoceran species (Chandini, 1989). A reduction in these levels may bring about food limitation, which in turn leads to undue stress, reduction in reproductive output and retardation of growth (Lanno *et al.*, 1989). For these reasons it is important to keep food levels high enough to obtain a significant feeding rate, but low enough to achieve the necessary algal depression. In this study, a reduction in algal concentration from 2.0×10^4 to 1.5×10^4 cells/mL was required as neonate animals have a substantially lower feeding rate than adults and do not require algal saturation (Table 4.2). In previous research an algal concentration of 1.5×10^4 cells per mL brought about increased sensitivity to cadmium in *Daphnia carinata* (Chandini, 1989). However, it is important to note that *D. carinata* is significantly larger than *M. macleayi* and cadmium sensitivities may be considerably different. A change of cell density did not significantly alter feeding rates of *M. macleayi* and the use of less algae also reduces laboratory workload. Thus the algal density used for all future testing with neonates was determined to be 1.5×10^4 cells/mL.

Just as algal cell density is an important factor in maintaining healthy populations of *M. macleayi* in the lab, food availability in the form of mixed natural food sources such as phytoplankton and bacteria are of equal importance to zooplankton life in the environment (Herbert, 1978). During seasonal and diurnal changes it is possible for food availability to fluctuate in the natural environment bringing about issues with increased sensitivity to toxicants with decreased ambient food levels. Due to the fact that it is too complex to mimic seasonal environmental food levels in the laboratory, it is important to account for the possibility of increased sensitivity both before and after release of waste waters.

4.5 Feeding test protocol (final)

Treatments (500 mL) were prepared in polyethylene bottles as per standard protocol (Section 2.7). Aliquots (50 mL) were dispensed into polyethylene nalgene bottles and acidified to 0.10 % for chemical analysis. The appropriate volume of *Chlorella* sp. was added to each bottle to produce an algal density of 2×10^5 cells/mL. Treatments were shaken and 60 mL aliquots were dispensed into 100 mL plastic vials for the measurement of pH, electrical conductivity and dissolved oxygen. Aliquots (15 mL) were dispensed into 45 mL replicate vials (7 per treatment). Each replicate vials with a letter corresponding to the treatment (e.g. A-G) and a replicate number (e.g. 1-7). An aliquot of filtered dilution water (15 mL) was also dispensed and labelled Coulter blank (CB). All vials were covered and placed in an incubator until they had reached 27 ∘C. Five *M. macleayi* neonates (<6 h) were transferred, into five of the seven vials per treatment, using a pasteur pipette, working from lowest concentration to highest concentration. The last two vials were the treatment blanks and did not contain animals. Samples were prepared from the treatment blanks for each treatment and Coulter counts taken using standard protocol to achieve T_0 counts (Appendix 10). Labelled lids, containg 2, two millimeter diameter holes were used to cover vials. Vials were placed on perspex trays (one per treatment) and incubated in a constant temperature incubator at 27∘C for 20 hours in the dark. Completion of this stage constitutes T_0. After 20 hours animals were removed and counts taken for each vial including the Coulter blank using standard Coulter conter protocol (Appendix 10). Test water was combined for each treatment and pH, conductivity and dissolved oxygen was measured. Cell density and feeding rates per animal were calculated using the formula:

$$F = \frac{V.\,(Ci - Cf)}{n.t}$$

F = Feeding rate in cells/animal/h

Ci = Initial cell concentration (T_{20} treatment blank counts)

Cf = Final cell concentration (T_{20} replicate counts)

V = Test volume in mL

n = No. of animals

t= Test duration

Acceptability of test data

The data set of the test was considered to be valid if:

The incubator temperature remains within 27 $\pm$ 1 ∘C;

Controls have at least 4 surviving neonates per replicate;

Changes in cell counts between T_0 and T_{20} are consistent for all treatments

Data sets for each group were considered acceptable if:

Three out of five replicates are valid

T_{20} pH is within 0.5 units of the T_0 pH

Dissolved oxygen is >70 % of the air saturation at a temperature of 27 ∘C

Chapter 5: Application

5.1 Introduction

The effects of exposure to single metals (i.e. cadmium, copper and uranium) on the feeding rate of *M. macleayi* have been suitably studied (Orchard, 1999; This study). However, less is known about the applicability of the test to predict the toxicity of heavy metal mixtures and organic compounds. The test was sensitive to gold mine release water however, failed to predict toxicity in uranium mine waste water (Orchard, 1999). The rapid nature of the test would favour its use from an industrial perspective and therefore it was considered necessary to determine the sensitivity of the test for a range of waste waters. Four waste waters, varying in primary metal/organic contaminants, were assessed and evaluations performed on each to determine the probable causes of toxicity. The four waste waters were:

1. Ranger Uranium Mine retention pond 2 waste water
2. Gold Mine A pit water
3. Gold Mine B tailings water
4. Jabiru Sewage Ponds influent and effluent

Retention pond II (RP2) water quality at Ranger mine is influenced by input from various sources of the Ranger water management systems. This includes runoff from the mine/mill area, stockpile catchment, transfer from Ranger #3 pit and recycled water (Figure 5.1, 5.2). Provision has been made for the release of RP2 water into the Magela Creek depending on water quality and hydrological criteria. However, this has not occurred thus far. Instead, primary output is by evaporation and use in the mine, mill and irrigation systems (ERA, 1999). Primary components of RP2 water for the test were, and have historically been, uranium, manganese and magnesium sulphate (Table 5.1 and 5.3). Gold mine A is an anonymous gold mine located in the Northern Territory of Australia. The pit receives tailing discharge and a natural inflow of groundwater (G. Parker, NTDME, pers. comm.). Cyanide has been used at the site in concentrations <5 ppm however, major contaminants were known to be arsenic, copper and zinc (Table 5.4).

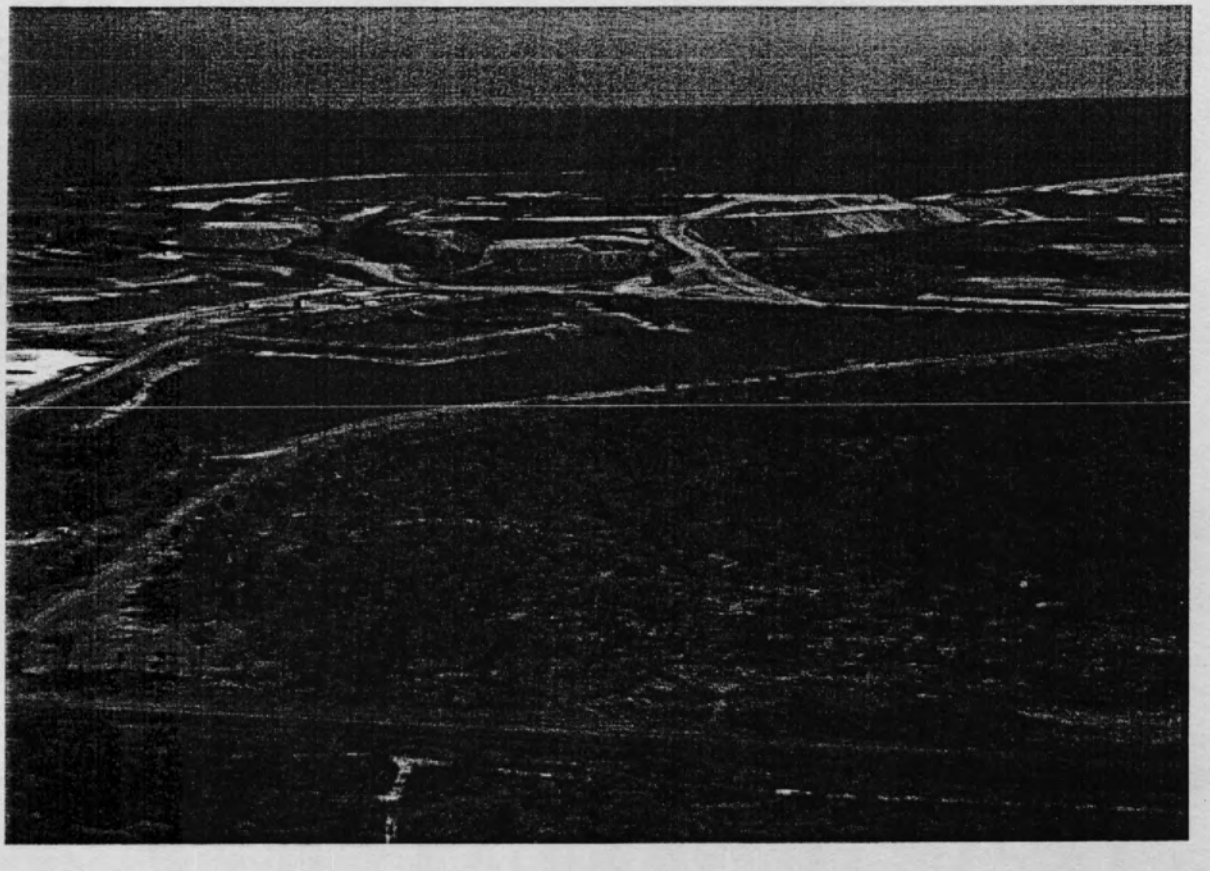


Figure 5.1: Aerial Photograph of Ranger Uranium Mine Retention Pond 2, August 2000

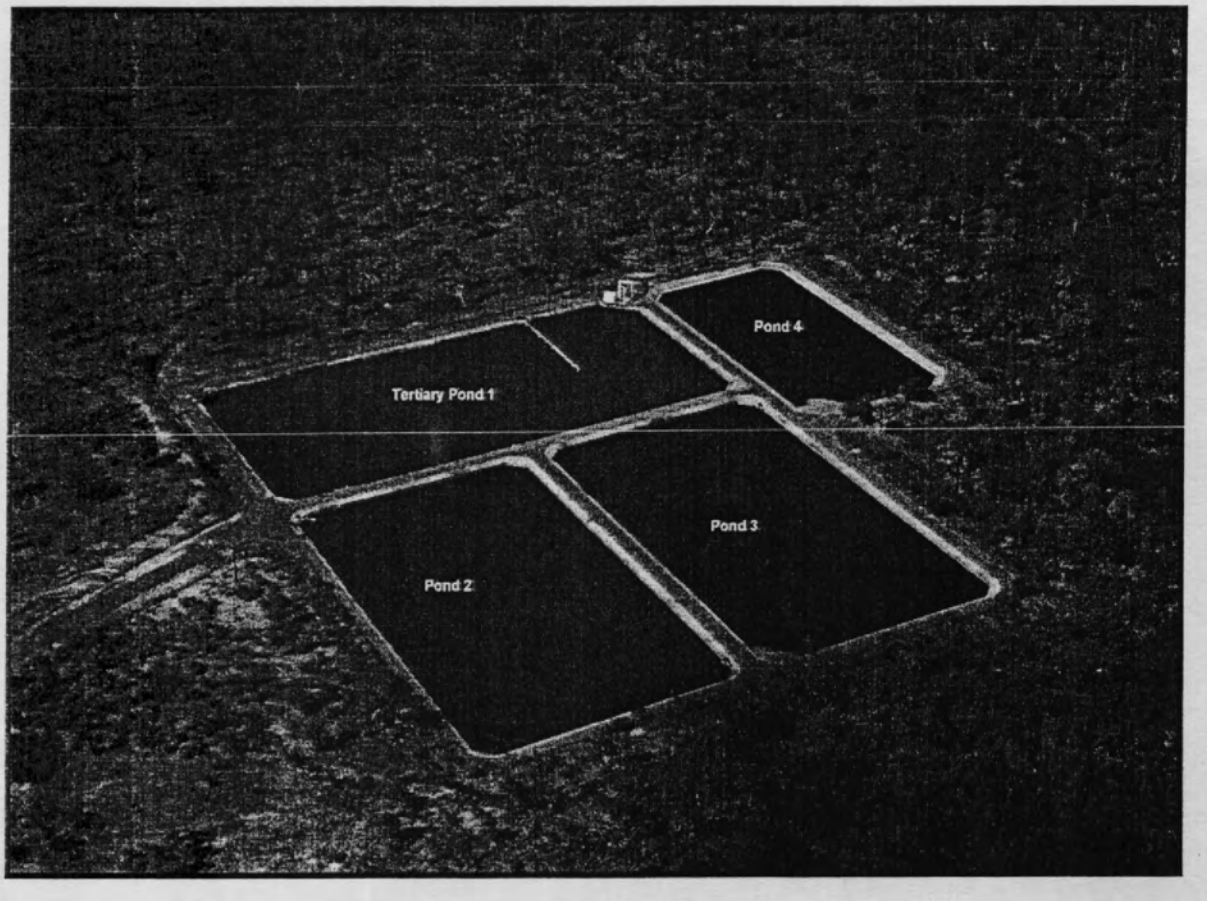


Figure 5.2: Aerial Photograph of Jabiru Sewage Treatment Ponds

Gold Mine B is an anonymous gold mine located in the Northern Territory. Tailings are sulphidic and are potentially acidic (G. Parker, NTDME, pers. comm.). From historical data it is known that the major components of the tailings decant are likely to be copper, lead, cadmium and manganese (Table 5.6) (G. Parker, NTDME, pers. comm.).

The Jabiru sewage treatment facility consists of a cycle of four ponds on a continuous flow system (Figure 5.3). A 28-day cycle is used to treat raw sewage with approximately seven days of treatment occurring in each pond. Final objectives of treatment are to achieve a biological oxygen demand of less than 20 %, and suspended solids less than 30 g/L. Settlement pond one has a primary intake of sewage from a current population of approximately 1200 people. Considering the wet dry climate of the area it is not viable for the facility to take in stormwater as this will lead to a system overload during wetter months. Primary contaminants were ammonia, nitrate, nitrite, heavy metals and surfactants (Table 5.8).

5.2 Methods

5.2.1 Ranger Uranium Mine retention pond 2 water

Retention pond 2 wastewater was collected by Energy Resources Australia (ERA) staff and refrigerated at 4 °C until required for further preparation. The sample was filtered through Whatman No. 42 grade filter paper (2.5 µm pore size) 24 hours after collection. Seven treatments were prepared using 0, 0.1, 0.3, 1.0, 3.0, 10, and 30 % RP2 water and filtered creek water from Bowerbird Billabong as the diluent. The feeding test was conducted using the final protocol (Section 4.5) and the reproduction and acute immobilisation using standard protocols (Section 2.10.2 and 2.10.3). Only two replicates were used for the acute immobilisation experiment due to a shortage of stock neonates upon test commencement. Chemical analysis was undertaken for a suite of metals.

5.2.2 Gold Mine A pit water

The Department of Mines and Energy (DME) staff collected samples from a pit, which had been partially backfilled with sulphidic waste rock. After collection, the sample was

refrigerated for five days at 4 °C until filtration through Whatman No. 42 grade filter paper (2.5 µm-pore size). Treatments were prepared at 0, 0.1, 0.3, 1.0, 3.0, 10 and 30 % pit water, with filtered creek water from Bowerbird Billabong as the diluent. These treatments were used to conduct concurrent reproduction (n=10), acute immobilisation (n=2) and feeding tests (n=5). A second set of tests were conducted with the same waste water seven days later, using a narrowed range of treatment concentrations (0, 0.03, 0.1, 0.3, 1.0 and 3.0 %). Replicate numbers for each acute immobilisation test were dependent on the numbers of stock animals remaining to start the test. In this case, two replicates were used for the first acute immobilisation test and three for the second. Standard protocols were followed for both reproduction and acute tests (Section 1.10), while feeding tests used final protocols (Section 4.5). Chemical analysis was undertaken for a suite of metals in this waste water (Appendix 5).

5.2.4 Gold Mine B tailings water

A sample from the tailing effluent of Gold Mine B was collected by DME staff refrigerated and filtered through Whatman No. 42 grade filter paper (2.5 µm-pore size). Six treatment concentrations were prepared at 0, 0.1, 0.3, 1.0, 3.2 and 10 % tailings water and filtered creek water from Bowerbird Billabong as the diluent. Concurrent reproduction (n=10), feeding (n=5) and acute immobilisation (n=3) tests were conducted; reproduction and acute immobilisation tests used standard protocols (Section 2.10) and feeding with the final protocol (Section 4.5). Chemical analysis on this waste water was undertaken for a suite of metals (Appendix 5).

5.2.4 Jabiru sewage influent

Sewage effluent from pond 4 contained a large component of invertebrate and plant life indicating that this water may not be toxic *to M. macleayi.* Therefore, a twenty-litre sample was collected from the inlet to sewage pond one at the Jabiru sewage treatment facility on 30/8/00 as influent was more likely to be toxic. The sewage influent sample was refrigerated at 4 °C for five hours and then filtered through Whatman No. 42 grade

filter paper (2.5 μm-pore size). Seven treatment solutions were prepared using 0, 0.3, 1.0, 3.2, 10, 32 and 100 % filtered sewage influent and filtered creek water from Bowerbird Billabong as the diluent. Concurrent reproduction (n=10), feeding (n=5) and acute immobilisation (n=2) tests were conducted; reproduction and acute immobilisation used standard protocols (Section 2. 12) and feeding with the final protocol (Section 4.5). Three 50mL samples were taken for chemical analysis and stored in amber bottles. One of the three samples was acidified to 0.1 % HNO_3. A sample of sewage effluent was taken from sewage pond 4 on 14/9/00 to monitor the levels of natural algae in the wastewater. This sample was filtered through 2.5 μm filter paper and the algal concentration in the sample was counted using the standard Coulter counter protocol (Appendix 10). Chemical analysis was undertaken for metals, suspected pesticides and sufactants (Appendix 5).

5.2.5 Statistical Analysis

Test data was analysed using Toxcalc® to derive LOEC, EC_{10}, EC_{20} and EC_{50} estimates. The Linear Interpolation method was used for reproduction and feeding tests and Maximum Likelihood Probit Analysis was used for acute immobilisation tests.

5.3 Results

5.3.1 Ranger Uranium Mine RP2 water

Feeding, reproductive and acute responses to varying concentrations of RP2 wastewater are presented in Figures 5.3 a, b and c respectively. Control values for each test are represented by a line and stated at the bottom of each graph. Both acute and reproductive results reflected a standard concentration-response curve, while the feeding response was interrupted at 1.0 %, due to a 72 % increase in feeding (Figure 5.3a). Total brood numbers also increased slightly in the reproduction test at 1.0 %, however this increase was not significantly different from controls (Figure 5.3b). No mortality was recorded whilst assessing reproductive effects. LOEC values for feeding and reproduction were observed at 0.3 and 10 %, respectively. Acute responses were not as sensitive as reproduction and evidence of this did not occur until 32 % (Figure 5.3c). Feeding test

Table 5.1: Historical values for RP2 minimum and maximum solute concentrations

Parameter		Unit	1997/98	1998/99
EC	Min	µS/cm	750	740
	Max		1300	1400
Mg	Min	mg/L	51	87
	Max		120	160
SO_4	Min	mg/L	270	180
	Max		700	720
U	Min	µg/L	150	340
	Max		3000	7300

Table 5.2: RP2 waste water point estimates (95% CI) for feeding, reproductive and acute endpoints

	EC10	EC20	EC50
Feeding	0.059 [a]	1.987[a]	22.50
	(0.01-7.83)	(0.00-10.50)	(NA)
Reproduction	2.41[a]	12.88[b]	>30
	(1.15-10.45)	(2.38-15.62)	(NA)
Acute Immobilisation	11.53[b]	14.61[b]	23.84
	(0.00-15.71)	(6.13-26.49)	(NA)

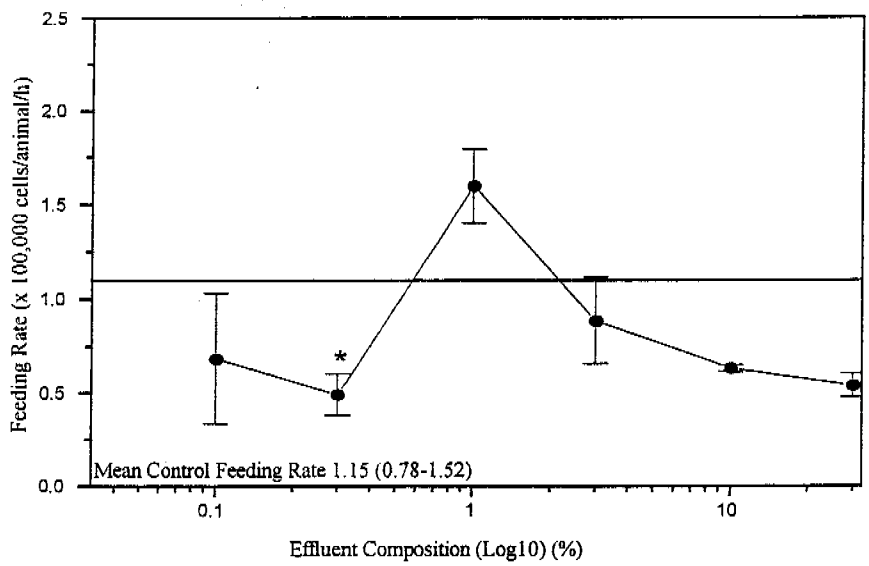
Linear interpolation used for analysis of all results
Comparisons are within columns where values with an alphabetical superscript in common are not significantly different (P>0.05)
NA: 95% Confidence Intervals not available

Table 5.3: Major components of RP2 water, 31/7/00

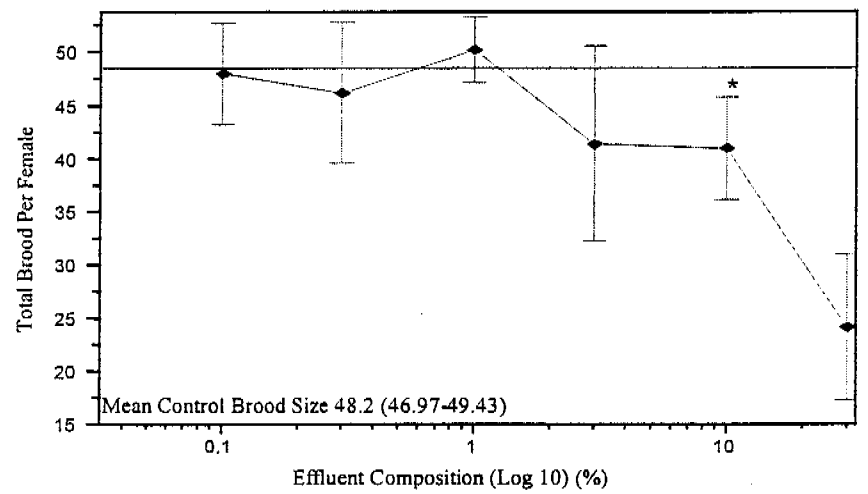
Element	Concentration (µg/L)
Al	138
Fe	420
Mg	131000
Mn	1420
SO4-S	531000
U	2237000

Further analysis is documented in Appendix 5

a)



b)



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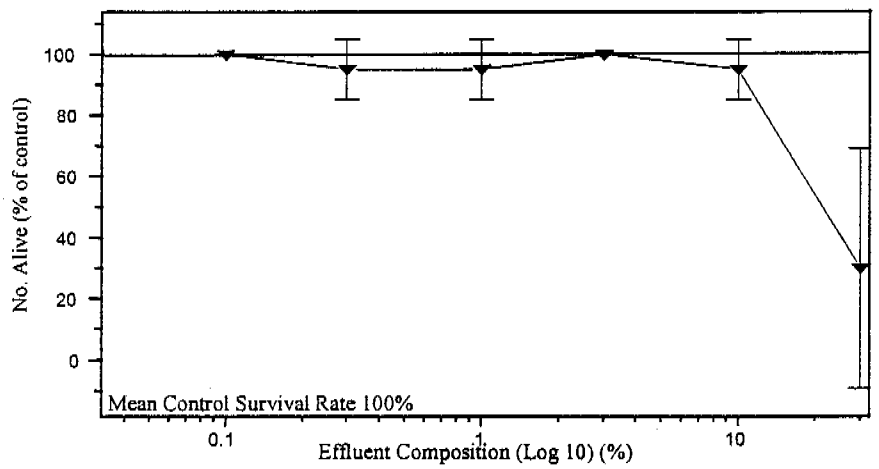


Figure 5.3: Effects of RPII waste water on *M. macleayi:* a)Feeding Rate (n=5)
b)Reproduction (n=10)
c)Acute Immobilisation (n=2)

Results are expressed as mean values (95% CI)

* LOEC with respect to controls

EC_{10} estimates were not significantly different from reproduction, while EC_{50} feeding estimates correlated closely to those of acute immobilisation. EC_{20} feeding estimates were significantly lower than those of reproduction and acute tests (Table 5.2).

5.3.2 Gold Mine A pit water

Feeding, reproductive and acute responses to Gold Mine A water are presented in Figures 5.4 a, b and c. Control values are represented by a line and stated at the bottom of each graph. Feeding sensitivity was evident in both feeding tests with LOEC values of 3 % and 0.1 % recorded for tests 1 and 2 respectively. Reproduction test 1 was discontinued due to a high proportion of male *M. macleayi* in controls. Reproduction test 2 recorded a 60 % death rate in control vials and therefore was invalid. There was no significant difference between EC_{50} estimates in the two acute immobilisation tests, with both being significantly higher than feeding EC_{50} estimates (Table 5.5).

5.3.3 Gold Mine B tailings water

Feeding, reproductive and acute responses to Gold Mine B tailings water are represented in Figures 5.5 a, b and c. Control values are represented by a line and stated at the bottom of each graph. An increase in feeding, from the LOEC of 0.03 % to 1.0 %, was observed that did not correspond to reproductive and acute responses. Point estimates indicated that the feeding inhibition was more sensitive than total offspring or survival rate (Table 5.7). However the calculation of estimates from an interrupted dose-response curve is usually inaccurate and therefore estimates may not provide an accurate representation of response.

5.3.4 Sewage Influent

Feeding, reproductive and acute responses are shown in Figures 5.6 a, b and c. Control values are represented by a line and stated at the bottom of each graph. A tri-modal feeding response was obtained due to a dramatic increase in feeding at 0.3 % and 10 %, which were significantly higher than the control feeding rate (Figure 5.5a). Due to a lack

Table 5.4: Major components of Gold Mine A pit water

Element	Test Sample
Zn (µg/L)	8760
Mn (µg/L)	37245
Mg (µg/L)	191978
Al (µg/L)	8099
Fe (µg/L)	3638
Sulphate (mg/L)	1737 (25/07/00)
As (µg/L)	59.3
Cu (µg/L)	389.95
Total Dissolved Solids (mg/L)	2546 (25/07/00)
Conductivity(µS/cm)	2920 (25/07/00)
pH	6.00 (25/07/00)

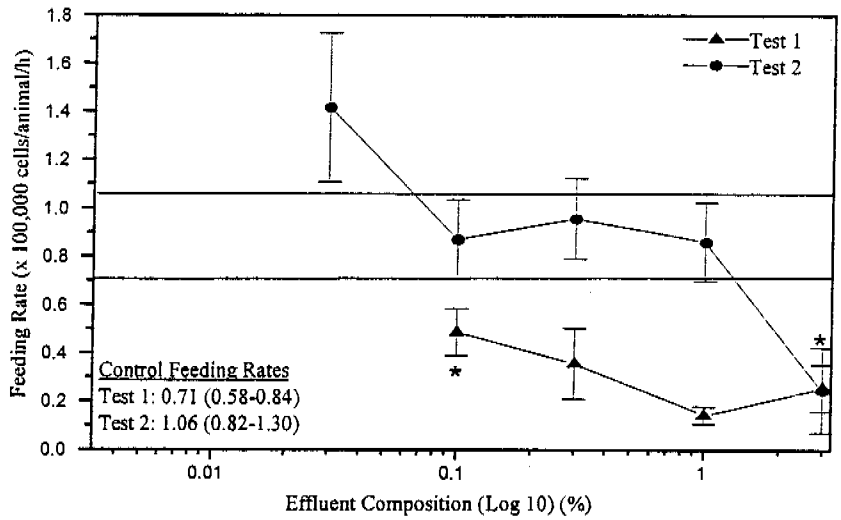
Further analysis is documented in Appendix 5

Table 5.5: Gold Mine A pit water point estimates (95% CI) for feeding, reproductive and acute endpoints

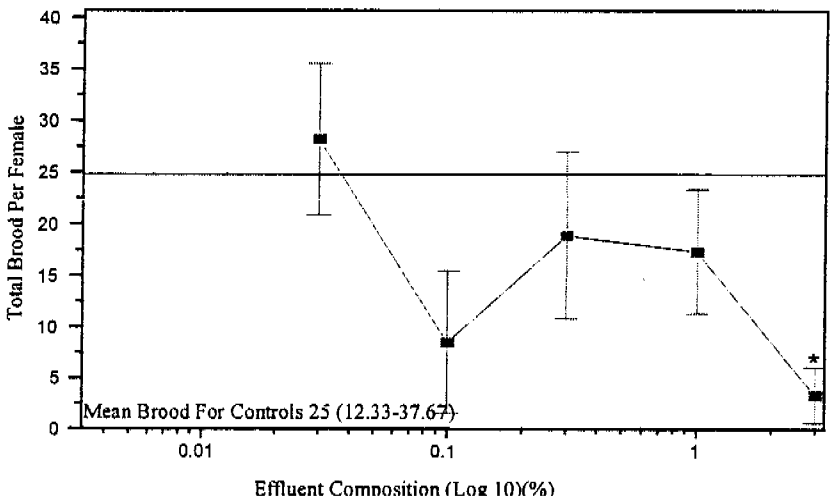
	EC_{10}	EC_{20}	EC_{50}
Feeding Test 1	0.03 (0.01-0.16)[a]	0.06 (0.02-0.20)[a]	0.31 (0.05-1.03)[a]
Feeding Test 2	0.05 (0.04-0.09)[a]	0.08 (0.06-1.61)[a]	1.79 (1.07-2.30)[b]
Reproduction Test 2 *	0.04 (0.01-0.06)[a]	0.06 (0.02-0.10)[a]	1.27 (0.07-1.96)[c]
Acute Test 1	NA	NA	2.61 (1.19-5.71)[c]
Acute Test 2	NA	NA	2.87 (0.89-9.25)[b,c]

EC_{50} estimates for acute tests were derived using the Trimmed Spearman-Karber method
Comparisons are within columns, where values with an alphabetical superscript in common are not significantly different ($p > 0.05$)
NA = Estimates cannot be derived *Invalid due to 60% control survival rate

a)



b)



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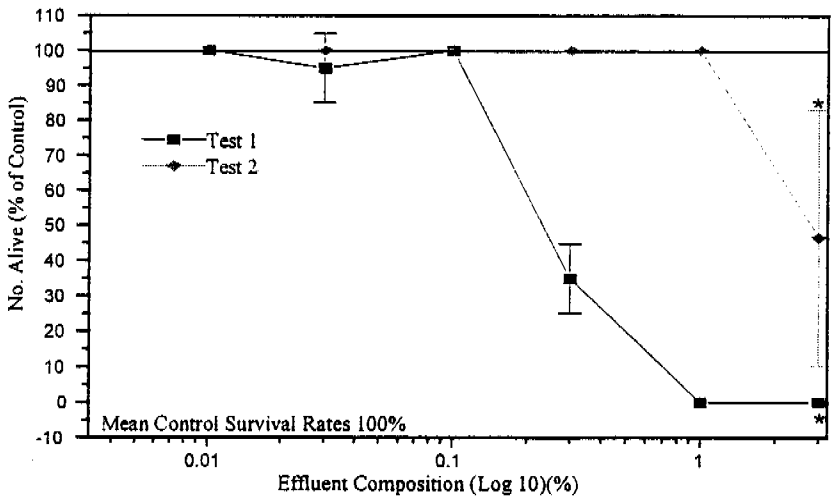


Figure 5.4: Effects of Gold Mine A waste water on *M. macleayi*: a) Feeding (n=5)
b) Reproduction (n=10)
c) Acute Immobilisation (n=2,3)

Results are expressed as mean values (95%CI). *LOEC with respect to controls.

Table 5.6 Major components of Gold Mine B tailings water

Component	Test Sample (µg/L)
Co	694
Cu	6409
Mg	77135
Mn	2498
Zn	113.1
Cd	0.4
Al	117
Rb	116
Pb	2.1
pH	8.3 (15/8/00)
EC (µS/cm)	3470 (15/8/00)

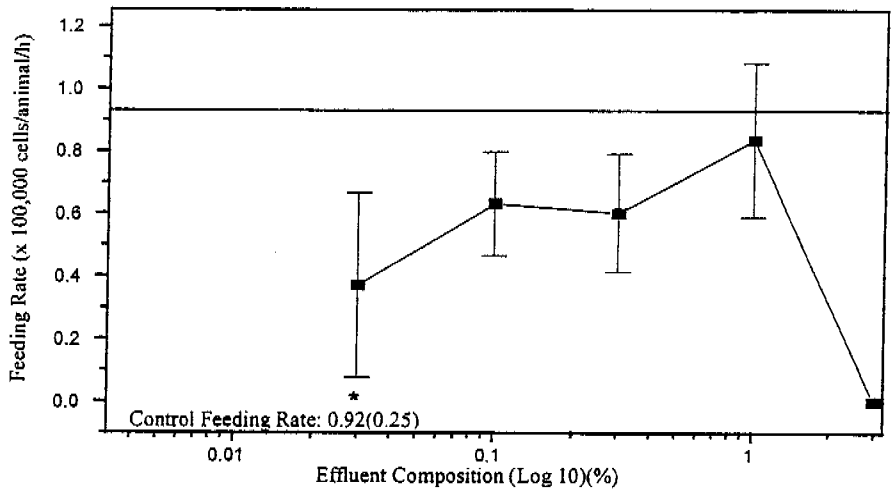
Further analysis is documented in Appendix 5

Table 5.7: Gold Mine B point estimates (95% CI) for feeding, reproduction and survival tests

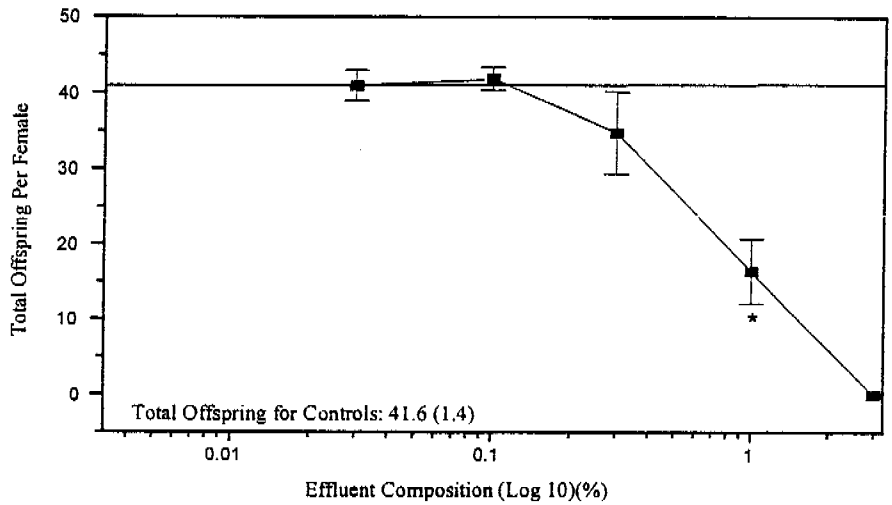
	EC_{10}	EC_{20}	EC_{50}
Feeding	0.01 (0.00-0.03)[a]	0.02 (0.01-1.81)[a]	1.50 (0.00-2.07)[a]
Reproduction	0.22 (0.15-0.37)[b]	0.36 (0.23-0.50)[a]	0.83 (0.70-0.95)[b]
Acute	0.94*	4.16*	5.00*

* 95% confidence intervals were not available for the acute immobilisation test
Comparisons are within columns where values with an alphabetical superscript in common are not significantly different ($p > 0.05$)

a)



b)



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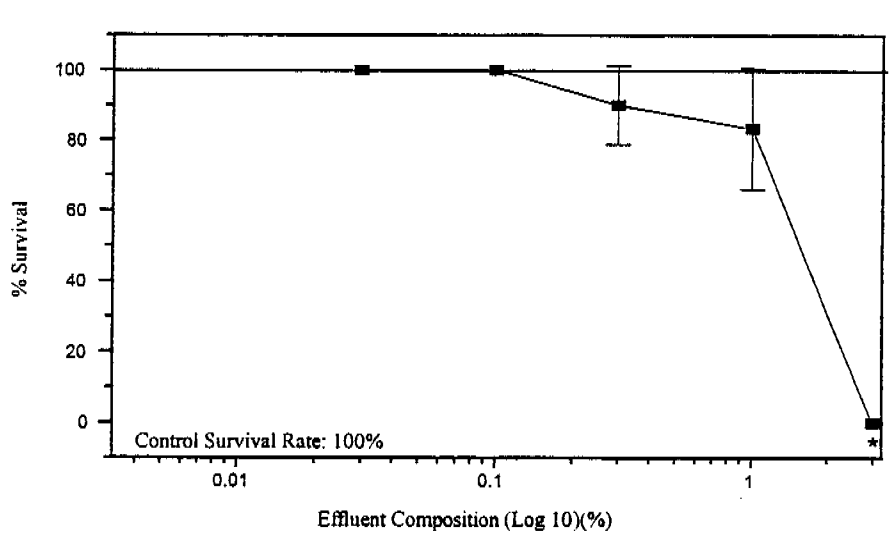


Figure 5.5: Effects of Gold Mine B tailings water to *M. macleayi*: a)Feeding (n=5)
b)Reproduction (n=10)
c)Acute Immobilisation (n=3)

Results are expressed as mean values (95% CI)
* Denotes LOEC

Table 5.8: Components of Sewage Influent

Component	Test Sample (µg/L)
Methyl blue active substances	4930
Cobalt thiocyanate active substances	2400
Diazinon	<1.0
Chloropyriphos	<1.0
Malathianon	<1.0
Mg	31100
Mn	23
Fe	166
Al	41
Cl	20000
$N - NO_3$	<5
$N - NH_3$	44900

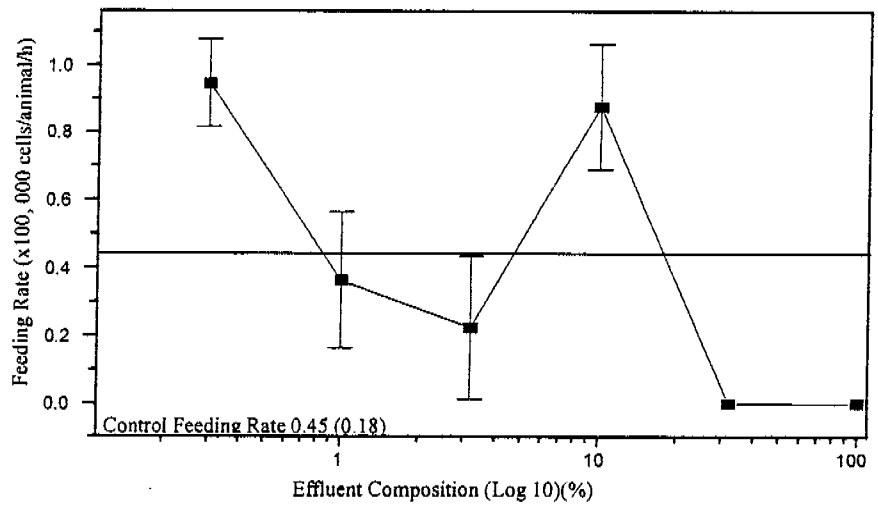
Further analysis is documented in Appendix 5

Table 5.9: Sewage Influent point estimates (95%CI) for feeding, reproductive and acute endpoints

	EC_{10}	EC_{20}	EC_{50}
Feeding	0.53 (0.41-0.95)	0.70 (0.52-16.88)	16.30 (8.92-20.64)
Reproduction*	0.05 (0.04-0.06)	0.09 (0.08-0.13)	0.24 (0.21-37.96)
Acute	NA	NA	56.56

*Reproduction test invalid due to a 40% death rate in controls
Acute results analysed using Trimmed Spearman-Karber
NA - Not available

a)



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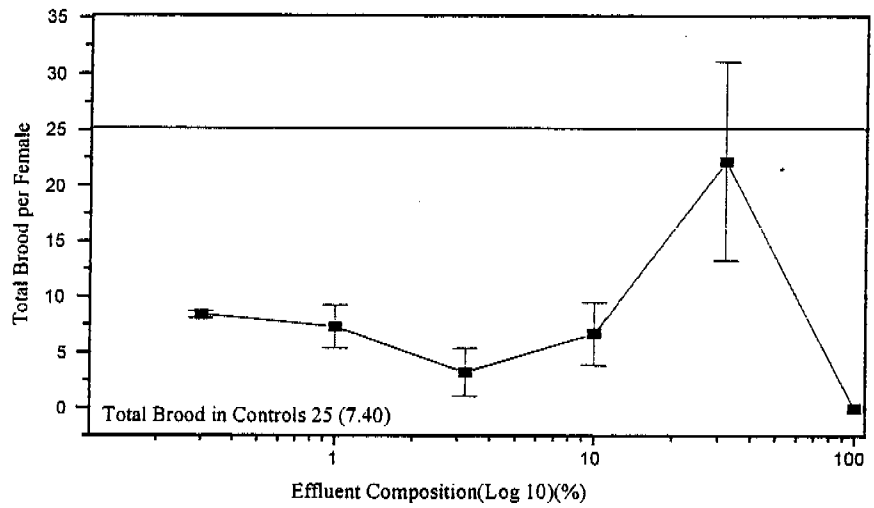

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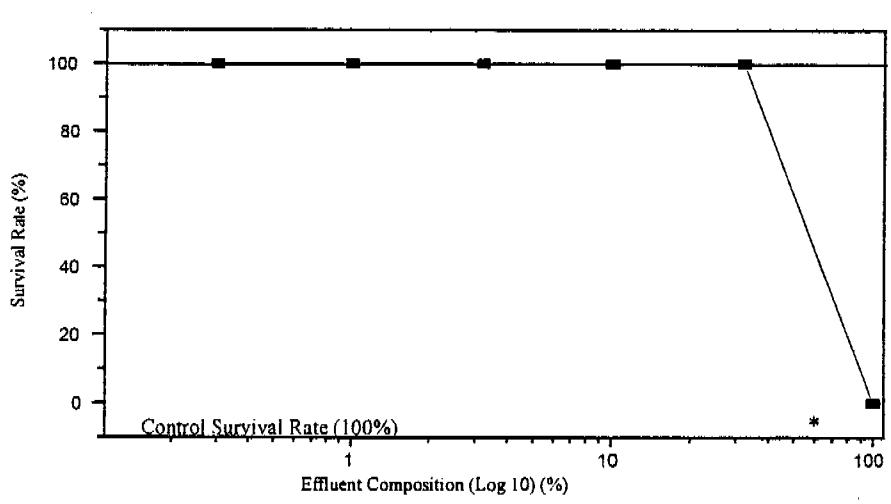

Figure 5.6: Effects sewage influent on *M. macleayi* a) Feeding Rate (n=5)
b) Reproduction (n=10)
c) Acute Immobilisation (n=2).

Results are expressed as mean values (95%CI)

of replicates and high mortality from 10 % to 30 % in the acute immobilisation test, it was not possible to calculate NOEC and LOEC values or perform probit analysis to obtain a complete array of point estimates. Feeding provided sensitive estimations of toxicity, more so than acute immobilisation, based on EC_{50} estimates. The accuracy of the feeding EC_{50} was questionable due to an interrupted concentration-response curve. Reproduction estimates could not be compared, as the test was considered invalid due to a 40 % death rate in controls. Background algal counts for the sewage effluent pond were 42×10^4 cell/mL.

5.4 Discussion

5.4.1 Ranger Uranium Mine RP2 water

Feeding response appeared sensitive to major RP2 contaminants (LOEC =0.3 %) and provided point estimates significantly lower than both the reproduction and acute immobilisation tests. However, further increases in feeding rate at higher concentrations indicated that a possible bi-modal response was obtained. It is possible that this increase was due to a range of non-feeding related factors (e.g. natural variability between animals) and therefore the authenticity of the feeding response was questionable. Feeding inhibition was considered an inappropriate indicator of toxicity for this waste water due to a huge variability in response. These findings correspond with previous results for Ranger Uranium Mine RP4 water, where an increase in feeding was observed at 3.2 % (Orchard, 1999). Both RP2 (This study) and RP4 (Orchard, 1999) feeding tests provided similar results, with feeding occurring at high concentrations of uranium (RP2 >0.3 % and RP4>1 %). Previous studies have also indicated that uranium has little impact on feeding rates, with an absence of feeding response recorded for uranyl sulphate at concentrations exceeding 300 µg/L (Orchard, 1999). Reproduction bioassays, assessing both RP2 (this study) and RP4 effluent (Orchard, 1999), have observed delayed reproductive effects, supporting previous reproductive studies of *M. macleayi* in response to uranium (Rippon, 1993; Semaan, 1999). Previous reproductive studies have recorded mortalities at uranium concentrations of 42 µg/L and reproductive impairment at

approximately 20 µg/L (Semaan, 1999). As no survival effects were observed in reproduction tests upon exposure of *M. macleayi* to 32 % RP2 waste water, which contained a uranium concentration of 716 µg/L, it is evident that uranium toxicity was decreased. Possible causes for this decrease are:

a) Complexing with dissolved organic matter.
b) Effects of pH, water hardness and alkalinity.
c) Complexing of the UO_2^{2+} ion with carbonate or phosphate (ANZECC, 2000).

In addition to uranium, manganese concentrations are frequently high in RP2 water. Manganese is a relatively non-toxic trace metal which can be concentrated up to four orders of magnitude by plants, microorganisms and animals (ANZECC, 2000). NOEC values for five representative crustacean species lie between 4.7 and 771 mg/L (ANZECC, 2000), in comparison to RP2 at 1.4 mg/L (31/7/00). Therefore, it is unlikely that this is the cause of toxic effects to *M. macleayi*.

Little research has been conducted on effect levels and mechanisms of toxicity for magnesium sulphate. However, at high concentrations magnesium may displace calcium ions hindering muscle formation and function in biota. In addition, at high salt concentrations (10 fold increase from natural salinity levels), maintenance of a constant ionic concentration is difficult. This results in the uptake of metal ions and the loss of water from cells, which may ultimately result in death (Hart *et al.*, 1991). These implications are not likely to have an impact on feeding results but may have implications for the longer duration tests such as reproduction and acute immobilisation.

Questionable feeding test results have now been obtained from two separate studies using uranium mine effluent, indicating that feeding inhibition is not a sensitive endpoint for effluents dominated by uranium.

5.4.2 Gold Mine A pit water

Both feeding tests were sensitive to gold mine waste water, although a major concern for the test was reproducibility of results as there were considerable differences in LOEC values for the "same" waste water (i.e. after seven days refrigeration). Small changes in waste water composition over time may result in small discrepancies between tests, although this was not likely to result in such large variations considering:

a) Metal speciation should not have changed significantly over the refrigeration period.
b) Minimal volatilization would occur due to low levels of cyanide and other volatile substances.
c) Degradation of organic components is reduced by refrigeration.

This change may be due to a decrease in the condition of *M. macleayi* stock cultures. Effective culturing of test animals requires the provision of fresh creek water, constant temperature and light regime, food supplements and fresh algae daily. Alteration to any one of these factors may affect the health and fecundity of the animals (*i.e.* male *M. macleayi* are produced under stressed conditions) and may lead to death. The first reproduction, feeding and acute tests were commenced with test animals that had been maintained on old (> 2 months) algae for three days prior to the start of the test. Reduced fitness of these animals may possibly be the cause of a reduced feeding rate, the production of males in control vials and increased mortality at lower concentrations in the first set of tests

A 60 % mortality rate in controls for reproduction test 2 was recorded, and therefore it was not possible to statistically compare feeding responses with reproductive responses for this waste water sample. Upon comparisons with acute data, feeding was a more sensitive response and produced EC_{50} estimates significantly lower than acute test 1. Previous results have indicated that feeding (LOEC=1 %) is a more sensitive indicator of toxicity than the reproduction test (LOEC=3.2 %) for gold mine release water (Orchard, 1999), although this would depend largely on the waste water composition. There are

likely to be similarities with primary contaminants between the waste water in this study and Orchard (1999) although these weren't discussed in the previous study. Gold Mine A waste water contained large amounts of copper, zinc, manganese, magnesium, aluminium and arsenic. However, metal concentrations above the levels known to cause effects to crustacean species were manganese, magnesium sulphate, copper and aluminium (Table 5.4).

Magnesium sulphate is known to have detrimental effects on cladoceran species, as discussed in Section 5.4.1, but these effects are not likely to cause feeding inhibition over a period of 20 hours. Copper is most likely to be the primary metal of concern in the wastewater; however it is not possible to rule out the effects of other principal metals and their interactions.

Levels of aluminium in the waste water were recorded at 8 099 µg/L which falls within the range of concentrations known to cause a 50 % mortality rate in Cladocera (2 300-36 900 µg/L – 1 species range) (ANZECC, 2000). This range is based on toxicity data from one species, which is an indication of the limited knowledge of aluminium toxicity to Cladocera. Prior knowledge of the sensitivity to metals of *M. macleayi* indicates that the possibility of a decrease in feeding at these concentrations cannot be eliminated (Table 1.2).

Manganese levels of 37 200 µg/L were recorded in the sample which are significantly higher than those causing 50 % mortality in *Daphnia magna* (4 700 µg/L)(ANZECC, 2000). Mechanisms of toxicity for manganese have not been studied extensively and therefore the possible feeding effects of the metal are unknown.

In summary, it is possible that the feeding response in the study is a result of copper, manganese and/or aluminium toxicity and/or interactions of these metals. Feeding inhibition is considered an appropriate endpoint for effluents with these primary

components based on comparisons with survival data. However, before any major conclusions can be drawn it is highly desirable to provide reproductive comparisons, and therefore further application of the feeding test to gold mine tailings water was conducted in Section 5.4.3.

5.4.3 Gold Mine B tailings water

Feeding did not provide an accurate estimation of toxicity of Gold Mine B tailings water when compared to reproductive and acute endpoints. Interruptions to the traditional concentration-response curve occurred when feeding increased at 0.03 and 1.0 % (Figure 5.6a). The first increase was suspected to be due to a hormetic effect, where as the second may have been energy related. For example, the animal required more energy for detoxification processes and therefore increases feeding to meet this energy demand. Until this response is investigated in more detail, no conclusions can drawn from these results.

Metals of ecotoxicological concern in the waste water included copper, cadmium, cobalt, nickel, zinc, molybdenum and manganese (Table 5.6). Copper is considered the dominant metal in the waste water at levels of 6 409 μg/L (NOEC for *Daphnia pulex* reproduction and survival, 1.7 μg/L). However, cadmium, cobalt, nickel, magnesium, molybdenum and manganese were also found in concentrations known to be toxic to cladoceran species. Thus, it is likely that the observed effects could not be attributed one metal, but a complex interaction between all metals. Vast interactions and complexations can occur within a waste water at varying levels of pH and water hardness (Erten-Unal *et al.*, 1998). The bioavailability of metals such as lead, zinc and cadmium will be increased by the high sulphate component in the sample (Table 5.6), as formation of metal salts will increase the solubility of these metals (Erten-Unal *et al.*, 1998).

The feeding test was a sensitive indicator of gold mine waste water toxicity in three out of four assessments from this study and Orchard (1999). However, it is also important to

note that gold mine wastes can vary greatly in composition, hence feeding effects may also vary greatly (as demonstrated by the feeding test for Gold Mine B waste water). Due to the unknown interactions of metals in waste waters, it is difficult to predict the bioavaliability of each metal or determine which metals are likely to cause feeding inhibition. Therefore more research to develop a knowledge of these factors so that accurate judgments can be made on the applicability of the feeding test to complex wastes.

5.4.3 Sewage Influent

Whilst the reproduction test was invalid, feeding and acute responses could still be compared. The feeding test did not provide an accurate estimation of toxicity for sewage influent in comparison to the acute immobilisation test. One factor limiting the use of the feeding test for sewage influent was a high content of dissolved organic matter in the sample. This led to an overall decrease in dissolved oxygen, due to the high demand for oxygen for decomposition (Appendix 2). Along with a reduction in dissolved oxygen, particulate matter in the samples resulted in a dramatic increase in Coulter counts. Since it was not possible to regulate the amount and size of the particles in each treatment, this factor may have had a significant impact on overall results. The sample may have also contained a large amount of bacteria, of which is also an important natural food source for *M. macleayi.* Instead of the animal reducing feeding as a result of toxic substances in the waste water, feeding rates of algae decreased due to consumption of an alternate food source that was not quantified by the Coulter counter. Hence, the feeding test may be unsuitable for effluents with a high proportion of particulate matter, bacteria and phytoplankton.

The reproduction test was invalid, due to a 40 % death rate in control animals, and therefore comparisons with this data could not be made. This death was later attributed to a reduction in health of adults due to poor food quality. The importance of nutrition to the

sensitivity of juvenile *D. magna* has been realised (Erten-Unal *et al.*, 1998), but due to time constraints the reproduction tests could not be repeated.

There were no metal concentrations of ecotoxicological importance in the sample. In addition, the possibility of any metal complexes being toxic would be dramatically decreased due to the high levels of dissolved organic matter. However, if the metal were to bind to algal particles the uptake of the metal through the gastrointestinal tract of the animal would be increased (Taylor *et al.*, 1998).

Diazinon and Malathion (pest control agents in flea and tick control shampoo for dogs), and chloropyriphos (termite control) were suspected to be present in the sewage influent. These substances were not detected in the waste, however this does not eliminate the possibility of occurence in other sampling events.

Cholorine levels were high (20 000 µg/L) in comparison to the 1 h *Ceriodaphnia dubia* LC_{50} of 280 µg Cl/L, and therefore may be responsible for some toxic effects in the influent. Although chlorine data was derived using *Ceriodaphnia dubia*, approximate levels are likely to be similar if not more sensitive for *M. macleayi* due to size differences. The source of the chlorine is unknown however, it may have originated from the treatment of drinking water.

At the time of sampling the major source of surfactants in Jabiru was most likely to be detergents from household products. Surface active substances (surfactants) are grouped into three groups; anionic (methyl blue active substances - MBAS), non-ionic (cobalt thiocyanate active substances - CTAS) and cationic substances (mainly quaternary ammonium compounds) (Bailey, 2000). Whilst the influent contained minimal levels of CTAS, MBAS levels were 49 300 µg/L. Trigger values (95% protection) for linear alkylbenzene sulphonates (LAS) and alkyl ethoxylated sulphonates (AES), two major components of MBAS, are defined to be 1 400-3 200 µg/L (2 species range) and 1 100 –

1 500 µg/L (*D. magna*) respectively. Therefore, MBAS were likely to contribute to toxicity in these tests. It is also important to note that trigger levels are derived for effluents for "safe" release into the environment. Jabiru sewage influent is not released into the environment until it has undertaken 21 days of treatment, over which a significant amount of substances break down or volatilise.

Total ammonia in the sample was determined to be approximately 44 900 µg/L and considering 24 h LC_{50} values for the Cladoceran *Simocephalus vetulus* are 1 580 µg/L, toxic impacts on *M. macleayi* are likely to be significant for this waste. At treatment water temperature (27 °C) and pH (7.0-7.7), the bioavailability of the ammonia is also likely to be increased due to a higher percentage of unionized ammonia (Appendix 9) (ANZECC, 2000). Since feeding rate and acute responses provided very different results, it is likely that the mechanisms of ammonia toxicity are different for each endpoint. However information on the mechanisms of ammonia toxicity is limited.

Further application of the test to treated sewage effluent was difficult due to a high level of background algae. As an indication, the background algal concentration in a filtered effluent sample (2.5 µm) from Jabiru sewage pond 4 (Figure 5.3) was 42.3×10^4 cells/mL which is over two times the concentration used for the feeding test. A range of natural algae assemblages are often found in sewage effluent and therefore the feeding test would not be suitable for such effluents, as it is not possible to restrict the feeding of *M.macleayi*.

Chapter 6: Summary

The use of feeding inhibition as a rapid and sensitive endpoint was investigated for the tropical cladoceran *Moinodaphnia macleayi*. This test was aimed at providing fast and accurate information on the toxicity of waste waters, in event of a chemical spill or need for waste water release. The initial protocol developed by Orchard (1999) was successfully reproduced in the Validation section, with *M. macleayi* producing a measurable feeding rate with the mean reduction in algal concentration for each replicate within the recommended range of 20-30 ± 10 %. Results were generally comparable with those of Orchard (1999).

In the Development section, the initial protocol (Section 2.10.1) was altered to derive a more reliable, sensitive and user-friendly test. Test volume was reduced from 30 mL to 15 mL, which also allowed for the use of *M. macleayi* neonates (< 6 h), instead of adults. The use of a 1:4 sample dilution allowed multiple samples to be taken from this volume, which is required in the event of a Coulter counter error. Neonate feeding rate was determined to be significantly lower than adult feeding rate and therefore the initial cell density was reduced from 2.0×10^5 to 1.5×10^5 cells/mL. Several problems were identified during the developmental process. Firstly it was determined that the Coulter counter could not distinguish algae from waste and therefore the counting range was narrowed to ensure that an accurate estimation of algal cell density was obtained. Secondly changes in the algal concentration of T_0 and T_{20} treatment blanks were observed, hence the feeding rate formula was altered to eliminate any day-to-day variation in counting procedure. Finally the practicality of the test was increased by the use of refrigeration to preserve samples for up to 20 hours. All of these changes were combined to create the final feeding test protocol (Section 4.5).

The validity of protocol to assess the toxicity of complex waste waters was then investigated in the Application section of the project. For each waste water, the feeding

test was compared to standard *M. macleayi* reproduction and mortality/immobilisation tests. The feeding response to Ranger retention pond water corresponded with previous findings by Orchard (1999) and confirmed that the feeding test was not suitable for waste water dominated by uranium. Promising results were obtained for two out of three feeding tests assessing gold mine waste water, with variability between tests possibly associated with different primary contaminants in the wastes. The test was not suitable for sewage influent nor effluent due to high particulate matter and the possibility of bacteria in the influent and high background algal counts in the effluent.

6.1 Limitations of this study and future research

Complex interactions occur in waste waters between metals, inorganic and organic matter and under these circumstances it is difficult to determine which components induce feeding effects. Obviously further application of the test is required to expand the knowledge of metal effects on feeding inhibition. A more thorough understanding of the types of toxicants known to cause feeding inhibition will lead to a greater understanding of the types of waste waters for which the feeding test may have application.

The investigation of irregular (bi- and tri-modal) feeding responses in the RP2 and Gold Mine B tests may outline some important aspects of energy allocation or mechanisms of toxicity to *M. macleayi*. An alternative to this would be to increase the test duration, as some metals have delayed mechanisms of toxicity and may not cause feeding effects within twenty hours. An extension of the test duration may obtain a response that would otherwise be overlooked. However, it is also important to note that the initial test was developed for rapid assessment and any alteration of test duration must not compromise this.

Several tests were conducted using animals reared on poor quality algae. Consequently, several reproduction tests were considered invalid due to an increased death rate. Reproductive results for the Gold Mine A and Sewage Influent would have allowed much

needed comparisons between the sub-lethal endpoints of feeding rate and reproduction. However, due to time constraints it was not possible to repeat these experiments.

Accurate measurement of algal concentration is an essential requirement of the test and the inability of the Coulter counter to distinguish algae from waste debris was a major limitation. Currently the feeding bioassay cannot be used for waste waters containing high quantities of particulate matter due to errors with Coulter counts. The alternative, manual cell enumeration, is time consuming and not practical for a rapid test. Therefore, methods for an alternate means of quantifying the amount of algae consumed (e.g. spectrophotomery) may need to be investigated in order to test these waste waters.

6.2 Conclusions

6.2.1 Feeding Test Validation

- Coulter counts did not correlate with microscope counts at T_{20} due to waste and debris being counted as "algae".

- A suitable feeding rate per animal was achieved, supporting the results of Orchard (1999) and the use of feeding inhibition as a favorable endpoint.

- The feeding test protocol demonstrated notable sensitivity to cadmium, therefore permitting its use as a reference toxicant in the following development section.

6.2.2 Development

- Changes in test sample volume, cell density and the use of *M. macleayi* neonates increased the practicality, reliability and sensitivity of the feeding test to cadmium.

- The accuracy of algal cell counts was not altered by changes to the counting range to 2.467-3.970 μm or the refrigeration of samples for up to 20 hours.

- The use of T_{20} counts as initial counts in Gauld's equation provided a more accurate representation of cadmium toxicity, as variability in counts due to Coulter counter changes and day-to-day test variation was eliminated.

6.1.3 Application

- The feeding test was not a suitable indicator of toxicity for Ranger Uranium Mine Retention Pond 2 water, supporting results obtained by Orchard (1999) for Retention pond 4 water.

- The feeding test could potentially predict the toxicity in gold mine waste waters, after two out of three feeding tests in this study, and one feeding test by Orchard (1999) demonstrated sensitivity to such waste waters.

- The feeding test was not suitable for the assessment of sewage influent or effluent due to the possible effects of particulate matter and bacteria in the influent and high background algae counts in the effluent.

6.1.1 Overall conclusion

At this stage the test was considered unsuitable to provide an accurate and reliable estimation of waste water toxicity due to large variations in feeding response and the subsequent lack of test reliability and reproducibility. However, the test appeared to have potential for gold mine waste waters, or waters with similar primary contaminants (i.e. copper, cadmium, zinc and aluminium). The test also has demonstrated sensitivity for some single toxicants such as cadmium. More research is required to determine which toxicants contribute to feeding inhibition and therefore whether a waste water might elicit a feeding response.

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Environment Australia

Department of the Environment and Heritage

Position Profile

Position Number:	**New**	**Date Approved:**
Designation:	EA Level 4	
Group/Division:	Supervising Scientist Division	
Branch:	Environmental Research Institute of the Supervising Scientist	
Section:	Ecosystem Protection	
State:	Jabiru, Northern Territory	
Immediate Supervisor:	EA Level 5	

DUTY STATEMENT

Under direction, contribute as a member of the EP Program's field-based monitoring unit. In particular:

1. Collect and sort benthic samples for macroinvertebrates and assist with identification and enumeration of constituent forms.
2. Organise and assist in the preparation of sampling trips to local and remote field sites, collect and process biological samples and undertake routine field measurements.
3. Undertake general laboratory duties and enter experimental/sampling data onto computer files and manipulate these data in preliminary analysis and graphics.
4. Purchase laboratory supplies and field equipment as required, and maintain neat and accurate records, including credit card and other purchase records.
5. Provide material, data and records to assist in the production of technical reports.
6. Participate actively in communications, employment and training programs, especially those that involve members of the local Aboriginal community.

Duty representing highest function: 1

SELECTION CRITERIA

Relevant knowledge, experience and skills (in order of importance):

1. Ability to carry out and perform accurately, laboratory and field experimental procedures; these may include repetitive and delicate tasks.
2. Experience in the preparation and general maintenance of field vehicles and boats, and provision of logistical support for field sampling.
3. Ability to follow written and verbal instructions.
4. Ability to maintain neat and accurate records.
5. Experience in ordering and purchasing laboratory and office supplies.

Personal attributes

1. Ability to live and work in a remote tropical locality and as part of a small team, including work in remote locations for periods of up to one week (and on occasions longer).
2. Driver's licence and experience with a range of vehicles.
3. Ability to work with a variety of people, including those from local communities and other stakeholder groups.

This position will transfer to the Jabiru Field Station when operational.

Appendix 1: Experiment log

Date	Project Section	Code	Test Name	Toxicant	Conc.	Diluent Water
3/5/00	Validation	535H	Clad Ref 2	None	1	Georgetown Coll: 24/3/00 Filt: 26/4/00
10/5/00	Validation	537H	Coulter Validation 1	None	1	Georgetown Coll: 24/3/00 Filt: 26/4/00
16/5/00	Validation	539H	Clad Feed Cd1	Cadmium	7	Georgetown Coll: 2/5/00 Filt: 12/5/00
17/5/00	Development	540D	Clad Culture	None	3	Georgetown Coll: 2/5/00 Filt: 8/5/00
21/5/00	Validation	541H,D	Clad Cd 2	Cadmium	7	Georgetown Coll: 2/5/00 Filt: 12/5/00
24/5/00	Development	542H	Clad Dev 1 Volumes	None	4	Georgetown Coll: 2/5/00 Filt: 16/5/00
1/6/00	Development	543H	Clad Dev 2 Neonates	None	6	Georgetown Coll: 2/5/00 Filt: 16/5/00
8/6/00	Development	544H	Clad Dev 3 Neonates 2	None	4	Georgetown Coll: 17/5/00 Filt: 7/6/00
8/6/00	Development	545H	Clad Dev 4 Dilution Factor	None	9	Georgetown Coll: 17/5/00 Filt: 7/6/00
8/6/00	Development	545H	Clad Dev 5 Coulter Validation Neonates	None	3	Georgetown Coll: 17/5/00 Filt: 7/6/00

Appendix 1: Experiment log (continued)

Date	Project Section	Code	Test Name	Toxicant	Conc.	Diluent Water
18/6/00	Development	546H,D	Clad Cd3	Cadmium	7	Georgetown Coll: 13/6/00 Filt: 16/6/00
2/7/00	Development	549H	Clad Dev 6 Coulter Ajustment	None	5	Georgetown Coll: 13/6/00 Filt: 29/6/00
6/7/00	Development	550H	Clad Cd 4	Cadmium	8	Georgetown Coll: 13/6/00 Filt: 5/7/00
6/7/00	Development	551H	Clad Dev 7 Coulter Ajustment 2	None	1	Georgetown Coll: 13/6/00 Filt: 5/7/00
13/7/00	Development	552H	Clad Cd 5 New Protocol	Cadmium	7	Georgetown Coll: 13/6/00 Filt: 5/7/00
16/7/00	Development	553H	Clad Cd 6 New Protocol	Cadmium	7	Bowerbird Coll:14/7/00 Filt: 16/7/00
23/7/00	Application	554H,D,I	Clad RP2	Ranger Uranium Mine RP2 Water	7	Bowerbird Coll:14/7/00 Filt: 19/7/00
9/8/00	Application	555H,D,I	Clad Gold Mine A	Gold Mine A Pit Water	7	Bowerbird Coll:14/7/00 Filt: 19/7/00
17/8/00	Application	556H,D,I	Clad Gold Mine A 2	Gold Mine A Pit Water	6	Bowerbird Coll:14/7/00 Filt: 19/7/00
31/8/00	Application	557H,D,I	Clad Sewage Influent	Jabiru Sewage Plant Influent	7	Bowerbird Coll:12/8/00 Filt: 13/8/00
10/9/00	Application	558H,D,I	Clad Gold Mine B	Gold Mine B Tailings Water	6	Bowerbird Coll:12/8/00 Filt: 30/8/00

Appendix 10: Coulter counter protocol

1. The Coulter counter is set to measure 500μL samples.
2. Before commencement of sample counts, several counts should be conducted using isoton solution. At this stage a counts should be below 800 cells/500μL, co-efficient of correction should be below 10 and counting time should be between 5.0-5.5 seconds.
3. The counts are then transferred to the window screen.
4. The range on the window screen should be set at 2.467-3.970μm.
5. The sample is then shaken and 2.5mL is dispensed into a 10mL volumetric flask.
6. The level in the flask is made up to the line (7.5mL) using isoton solution and the flask shaken three times.
7. The sample/isoton solution is transferred to a homogeniser and mixed using four twisted plunges of a pestle.
8. The solution is transferred to a 10mL plastic vial and a series of four consistent counts are taken.
9. Only counts within ± 5% of other counts and within ± 5% of the established time duration should be included.
10. If a prepared sample is let stand for more than three minutes before counting it should be re-homogenised.
11. Rinse all equipment with Milli Q water and repeat for the remaining samples.

Important checks:

1. Isoton resevoir is at least half full
2. Isoton waste jar is empty at the start of counting
3. Aperature is clear for each count
4. Mercury level in the manometer is approxiamtely one centimetre above the visible bulb in the viewing window on the sampling stand. Levels can be ajusted using the fine vacum control.
5. Both the Coulter counter and sampling stand are reset before the commencement of a new count

Appendix 2: Creek water collection dates and parameters

Collection Date	Location	Date Filtered	Water Parameters					
			Unfiltered			Filtered		
			pH	Cond. (µS/cm)	D.O (%)	pH	Cond. (µS/cm)	D.O (%)
24/3/00	Georgetown	26/4/00	6.28	19.3	88.0	6.49	18.4	80.0
2/5/00	Georgetown	8/5/00	6.45	18.9	95.0	6.62	18.3	82.0
2/5/00	Georgetown	12/5/00	6.71	21.5	88.0	6.68	17.7	100.0
2/5/00	Georgetown	16/5/00	6.60	21.0	100.5	6.81	19.3	89.6
17/5/00	Georgetown	7/6/00	6.66	24.3	88.3	6.69	20.1	99.5
13/6/00	Georgetown	13/6/00	6.56	19.3	101.0	6.75	20.5	87.0
13/6/00	Georgetown	16/6/00	6.64	20.1	100.5	6.82	21.5	93.0
13/6/00	Georgetown	29/6/00	6.69	21.8	101.5	6.86	17.8	91.0
13/6/00	Georgetown	5/7/00	6.82	29.2	102.6	6.92	22.1	104.7
14/7/00	Bowerbird	15/7/00	6.34	18.1	88.6	6.52	14.5	88.2
14/7/00	Bowerbird	19/7/00	6.39	16.3	90.2	6.51	19.1	89.5
11/8/00	Bowerbird	13/8/00	6.60	19.7	94.3	6.51	20.7	99.0
11/8/00	Bowerbird	17/8/00	6.56	18.9	101.5	6.34	19.4	102.3
11/8/00	Bowerbird	30/8/00	6.44	19.1	100.6	6.68	17.3	94.0

Appendix 3: Test water parameters

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
Clad Ref 2	H	4 Adults in 30mL Filtered Creek Water	6.7	19.5	98.5	6.8	23.6	98.5
Clad Coulter Validation 1	H	Filtered Creek.Water	6.5	22.1	85.6	6.7	29.7	98.6
		Filt. Ck. Water + Chlorella @ $2x10^5$ cells/mL	6.7	19.5	83.4	6.9	28.0	99.5
		4 Adults in 30mL Filtered Creek Water	6.7	19.5	83.4	6.9	28.0	99.5
Clad-Cd1	H	0µg/L Cd	6.7	19.7	91.7	6.8	23.6	102.5
		1.7µg/L Cd	6.8	18.8	97.4	6.8	20.7	103.6
		2.7µg/L Cd	6.9	18.8	96.6	6.9	20.6	103.4
		4.5µg/L Cd	6.9	19.0	96.5	6.9	20.6	104.3
		8.1µg/L Cd	6.9	18.0	98.5	6.9	19.4	103.3
		13.7µg/L Cd	6.9	18.6	98.2	6.9	18.7	105.5
		25.8µg/L Cd	6.8	18.9	98.6	8.5	26.7	103.6
Clad Culture	D	1 Animal per 30mL Filtered Creek Water	6.9(0.0)	27.4(2.0)	100.9(1.5)	7.3(0.6)	30.6(5.0)	99.5(1.3)
		2 Animals per 30mL Filtered Creek Water				7.3(0.3)	27.1(2.9)	99.0(0.9)
		3 Animals per 30mL Filtered Creek Water				7.2(0.3)	24.4(2.5)	100.3(2.2)
Clad Cd2	H	0µg/L Cd	6.9	20.2	99.6	7.35	21.3	98.5
		1.4µg/L Cd	6.9	18.7	102.4	6.83	23.1	100.6
		2.7µg/L Cd	6.9	23.1	102.5	6.95	20.7	99.7
		4.4µg/L Cd	6.9	19.7	100.6	7.02	21.4	98.5
		7.9µg/L Cd	6.9	18.2	101.1	6.98	40.5	98.7
		14.0µg/L Cd	6.9	18.3	101.5	6.99	21.7	99.2
		27.0µg/L Cd	6.9	18.6	103.6	6.95	23.8	98.2
	D	0µg/L Cd	6.8(0.2)	26.8(3.4)	105.5(2.5)	7.5(0.6)	24.6(2.6)	97.9(0.7)
		1.4µg/L Cd	6.8(0.3)	24.5(2.8)	107.0(2.7)	7.9(0.5)	22.7(1.0)	98.9(2.1)
		2.7µg/L Cd	6.9(0.2)	23.6(0.7)	107.0(2.7)	7.9(0.5)	23.6(0.9)	98.8(1.5)
		4.4µg/L Cd	6.9(0.2)	22.8(0.6)	166.8(2.7)	7.4(0.1)	22.4(0.6)	97.3(1.9)
		7.9µg/L Cd	6.9(0.1)	23.0(0.9)	107.1(3.2)	7.6(0.1)	22.5(0.9)	98.1(2.2)
		14.0µg/L Cd	6.9(0.2)	22.6(0.6)	104.5(4.2)	7.9(0.2)	22.2(0.3)	96.1(4.3)
		27.0µg/L Cd	6.9(0.2)	22.2(0.3)	22.2(0.3)	7.6(0.3)	22.5(0.1)	96.2(0.4)

Appendix 3: Test water parameters (continued)

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
Clad Dev 1 Volumes	H	4 adults in 30mL	6.79	57.9	99.5	7.01	21.6	93.7
		2 adults in 15mL	6.63	20.9	99.6	6.68	23.5	99.3
		2 adults in 10mL	6.68	17.5	99.6	6.83	19.9	99.3
		3 adults in 10mL	6.69	17.6	101.2	6.90	22.4	98.2
			6.69	17.6	101.2	6.92	22.5	101.1
Clad Dev 2 Neonates	H	0 neonates in 15mL	6.46	20.1	97.7	6.57	23.6	99.6
		3 neonates in 15mL				6.75	23.1	99.5
		4 neonates in 15mL				6.84	19.8	99.3
		5 neonates in 15mL				6.90	19.3	98.3
		6 neonates in 15mL				6.92	19.9	99.6
Clad Dev 3 Neonates 2	H	3 neonates in 15mL	6.53	22.3	98.2	6.57	24.7	98.6
		4 neonates in 15mL				6.75	21.7	99.5
		5 neonates in 15mL				6.91	23.2	98.5
		6 neonates in 15mL				6.81	21.8	99.3
Clad Cd 3	H	0µg/L Cd	6.9	24.6	102.1	6.64	28.6	104.6
		1.5µg/L Cd	6.9	25.8	101.2	6.81	19.6	101.6
		2.8µg/L Cd	6.8	31.2	100.6	6.96	32.9	101.7
		4.6µg/L Cd	6.8	22.8	100.7	6.96	19.1	100.5
		8.5µg/L Cd	6.8	22.7	101.3	6.94	18.7	99.7
		14.6µg/L Cd	6.8	22.8	101.7	6.91	18.4	100.5
		27.4µg/L Cd	6.8	22.6	101.4	6.85	18.2	100.5
	D	0µg/L Cd	6.6 (0.2)	27.8 (3.4)	103.4 (1.5)	7.7 (0.6)	25.0 (3.4)	98.2 (3.5)
		1.5µg/L Cd	6.8 (0.1)	21.8 (2.8)	101.2 (3.1)	7.9 (0.4)	25.7 (3.2)	97.5 (1.6)
		2.8µg/L Cd	6.8 (0.0)	25.7 (6.1)	102.2 (2.0)	7.9 (0.5)	23.8 (1.7)	97.7 (1.3)
		4.6µg/L Cd	6.8 (0.0)	21.2 (2.2)	101.7 (2.3)	7.5 (0.2)	23.8 (1.6)	95.5 (0.8)
		8.5µg/L Cd	6.8 (0.1)	20.9 (2.1)	102.0 (2.6)	7.6 (0.5)	24.4 (1.0)	96.3 (1.3)
		14.6µg/L Cd	6.8 (0.0)	19.9 (2.5)	101.0 (0.6)	7.9 (0.5)	24.0 (1.7)	96.1 (0.2)
		27.4µg/L Cd	6.8 (0.0)	19.7 (2.5)	100.1 (1.5)	7.7 (0.5)	25.7 (1.3)	94.7 (0.7)

Appendix 3: Test water parameters (continued)

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
Clad Cd 4	H	0µg/L Cd	6.6	23.6	100.1	6.8	21.3	95.5
		1.4µg/L Cd	6.7	19.4	100.5	7.0	20.5	96.2
		2.88µg/L Cd	6.9	19.2	99.8	6.9	21.2	96.5
		5.22µg/L Cd	6.8	18.9	99.5	7.0	18.6	99.1
		8.47µg/L Cd	6.8	18.5	100.1	7.1	18.5	98.2
		15.8µg/L Cd	6.8	19.6	102.0	7.1	18.2	95.3
		20.9µg/L Cd	6.87	17.9	97.5	6.69	18.1	96.3
		29.0µg/L Cd	6.87	18.2	98.2	6.73	18.0	97.4
Clad Cd 5 New Protocol	H	0 µg/L Cd	6.9	20.9	93.7	7.0	21.3	95.5
		1.8µg/L Cd	6.9	20.0	95.3	6.9	20.5	96.2
		3.0µg/L Cd	6.9	20.6	96.5	6.9	21.2	96.5
		4.8µg/L Cd	6.9	18.9	98.5	6.8	18.6	99.1
		9.2µg/L Cd	6.8	18.4	97.0	6.8	18.5	98.2
		16.3µg/L Cd	6.9	18.3	95.6	6.7	18.2	95.3
		28.6µg/L Cd	6.87	17.9	97.5	6.69	18.1	96.3
Clad Cd 6 New Protocol	H	0µg/L Cd	6.5	19.3	88.7	6.5	24.1	91.5
		1.5µg/L Cd	6.7	18.3	91.1	6.7	20.3	92.0
		3.0µg/L Cd	6.8	18.9	92.0	6.7	21.5	92.7
		5.4µg/L Cd	6.8	18.3	93.0	6.8	21.6	93.4
		9.5µg/L Cd	6.8	18.0	91.7	6.9	20.8	92.6
		22.2µg/L Cd	6.8	19.0	89.3	6.9	20.3	91.7
		27.5µg/L Cd	6.8	18.5	91.5	6.9	23.4	92.5
Clad RP2	H	0%	6.4	20.8	102.0	6.7	20.3	99.2
		0.1%	6.6	18.8	101.2	6.7	22.6	99.6
:		0.3%	6.6	21.6	101.0	6.9	42.2	98.6
		1.0%	6.6	30.3	102.0	6.8	32.1	99.0
		3.0%	6.6	56.9	101.0	6.9	57.7	99.0
		10%	7.0	146.2	99.2	6.9	143.8	96.6
		30%	7.2	386.0	98.6	7.2	376.0	99.5

Appendix 3: Test water parameters (continued)

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
Clad RP2	D	0%	6.7(0.3)	20.0(3.0)	103.6(3.3)	6.9 (0.3)	23.3(4.3)	95.1(2.4)
		0.1%	6.8(0.4)	19.2(1.7)	105.8(3.3)	7.1(0.2)	34.1(3.5)	94.1(2.4)
		0.3%	6.7(0.1)	21.0(0.7)	104.1(3.5)	7.1(0.1)	26.6(3.2)	95.1(2..8)
		1.0%	6.7(0.1)	31.0(0.5)	105.2(2.8)	7.0(0.1)	38.6(4.8)	94.7(3.1)
		3.0%	6.7(0.0)	60.5(2.0)	106.5(4.1)	7.0(0.1)	51.4(23.4)	94.5(2.6)
		10%	6.7(0.2)	147.0(0.8)	107.9(5.3)	7.8(0.1)	153.2(5.4)	95.1(2.9)
		30%	6.9(0.2)	388.4(1.7)	106.0(5.1)	7.2(0.1)	396.3(6.6)	93.4(3.0)
	I	0%	6.4	20.8	102.0	6.7	20.3	99.2
		0.1%	6.6	18.8	101.2	6.7	22.6	99.6
		0.3%	6.6	21.6	101.0	6.9	42.2	98.6
		1.0%	6.6	30.3	102.0	6.8	32.1	99.0
		3.0%	6.6	56.9	101.0	6.9	57.7	99.0
		10%	7.0	146.2	99.2	6.9	143.8	96.6
		30%	7.2	386.0	98.6	7.2	376.0	99.5
18.Clad Gold Mine A	H	0%	6.75	15.6	101.6	6.66	24.2	97.2
		0.1%	6.71	26.2	99.6	6.76	22.2	100.0
		0.3%	6.67	30.5	99.4	6.86	32.3	99.2
		1.0%	6.59	64.0	98.4	6.65	63.8	99.6
		3.0%	6.27	159.7	98.0	6.51	159.5	98.0
		10%	5.58	435.0	93.6	5.95	423.0	95.5
		30%	5.01	1119.0	95.2	5.01	1157.0	96.6
	I	0%	6.85	17.7	101.6	6.73	17.6	100.6
		0.1%	6.57	21.0	100.5	6.80	20.6	105.6
		0.3%	6.41	46.2	103.0	6.85	37.3	98.6
		1.0%	6.35	66.8	100.6	6.71	66.3	102.7
		3.0%	6.31	157.3	101.6	6.36	159.0	95.7
		10%	5.53	446.0	102.4	5.63	437.0	100.6
		30%	5.02	1124.0	100.5	4.81	1110.0	100.2

Appendix 3: Test water parameters (continued)

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
19. Clad Gold Mine A2	H	0%	6.58	21.3	129.4	6.61	28.5	94.6
		0.03%	6.63	18.5	131.5	6.80	32.1	96.2
		0.1%	6.68	21.2	128.3	6.83	39.1	94.0
		0.3%	6.59	31.2	132.3	6.75	46.7	91.3
		1.0%	6.57	66.6	130.6	6.73	75.8	90.5
		3.0%	6.27	156.6	133.5	6.47	155.0	91.5
	D	0%	6.8(0.2)	19.2(2.4)	103.3(15.9)	6.9(0.2)	19.9(2.4)	95.1(8.7)
		0.0%3	6.8(0.2)	18.6(0.7)	104.0(16.8)	7.0(0.1)	21.7(2.40	95.3(10.3)
		0.1%	6.7(0.1)	21.5(0.8)	100.6(15.5)	6.9(0.2)	24.9(2.5)	93.1(9.5)
		0.3%	6.6(0.2)	33.2(5.0)	103.9(16.8)	6.9(0.1)	35.9(3.7)	95.6(10.8)
		1.0%	6.6(0.1)	66.2(1.9)	100.6(16.0)	6.9(0.2)	71.9(9.6)	93.9(8.7)
		3.0%	6.4(0.20	157.8(3.7)	106.4(19.8)	6.7(0.3)	163.0(8.8)	93.4(9.9)
	I	0%	6.58	21.3	129.4	7.06	18.2	82.5
		0.03%	6.63	18.5	131.5	7.08	21.1	80.6
		0.1%	6.68	21.2	128.5	7.03	23.2	80.5
		0.3%	6.59	31.2	132.3	6.97	36.1	80.0
		1.0%	6.57	66.6	130.6	6.78	69.1	81.2
		3.0%	6.27	156.6	133.5	6.63	159.0	80.3
20. Clad Sewage Influent	H	0%	6.5	16.9	101.6	7.6	18.0	NR
		0.3%	6.6	18.0	100.1	7.4	19.9	
		1.0%	6.9	23.6	94.3	7.3	26.0	
		3.2%	6.8	42.3	95.9	7.6	47.2	
		10%	7.3	99.9	90.1	7.8	97.1	
		32%	7.7	276.0	84.1	8.3	252.0	
		100%	7.75	798.0	39.4	NR	NR	

Appendix 3: Test water parameters (continued)

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
Clad Sewage Influent	D	0%	7.0(0.5)	19.4(2.2)	90.4(10.5)	7.0(0.3)	23.6(2.5)	97.7(4.3)
		0.3%	6.9(0.3)	20.1(2.8)	89.8(8.7)	7.0(0.2)	25.0(2.6)	97.8(3.3)
		1.0%	6.9(0.1)	24.8(1.1)	81.8(11.7)	7.4(0.4)	30.3(2.0)	98.0(5.0)
		3.2%	6.9(0.1)	42.1(2.4)	75.5(15.6)	7.5(0.1)	50.2(2.7)	95.9(2.2)
		10%	7.5(0.1)	103.7(3.4)	67.4(18.5)	7.9(0.2)	105.9(1.2)	92.4(7.0)
		32%	7.7(0.1)	277.0(3.4)	54.3(26.7)	8.3(0.1)	261.2(25.4)	81.7(12.3)
		100%	7.7(0.0)	798.0(0.0)	39.4(0.0)	NR	NR	NR
	I	0	6.5	16.9	101.6	6.8	20.1	60.7
		0.3	6.6	18.0	100.1	6.9	19.1	42.6
		1.0	6.9	23.6	94.3	7.0	24.4	42.0
		3.2	6.8	42.3	95.9	7.3	42.2	51.5
		10	7.3	99.9	90.1	7.96	101.4	52.5
		32	7.7	276.0	84.1	8.27	272.0	39.7
		100	7.7	798.0	39.4	8.52	744.0	14.2
21. Clad Gold Mine B	H	0%	6.60	34.7	109.4	6.73	48.5	NR
		0.0%3	6.64	34.0	106.1	6.85	37.2	NR
		0.1%	6.60	37.6	109.7	6.88	39.5	101.5
		0.3%	6.64	45.4	109.2	6.86	47.1	103.6
		1.0%	6.88	69.6	108.3	6.92	68.4	101.4
		3.0%	6.77	152.3	107.3	NR	NR	NR
	D	0%	6.7(0.1)	33.6(1.0)	122.4(18.0)	7.1(0.2)	37.4(0.7)	106.2(12.9)
		0.0%3	6.8(0.1)	34.1(6.6)	121.3(17.0)	7.2(0.6)	39.7(2.6)	105.0(13.6)
		0.1%	6.7(0.1)	39.2(3.5)	119.2(15.5)	7.3(0.5)	41.8(0.6)	106.5(13.7)
		0.3%	6.7(0.1)	43.8(3.8)	119.0(15.0)	7.3(0.5)	49.7(1.4)	104.3(12.8)
		1.0%	6.8(0.1)	69.2(1.1)	118.1(19.8)	7.3(0.5)	72.9(1.4)	105.4(14.0)
		3.0%	6.7(0.0)	152.3(0.0)	107.3(0.0)	7.7(0.0)	154.3(0.0)	103.1(0.0)

Appendix 3: Test water parameters (continued)

Test Name	Type	Treatment/Toxicant Concentrations	Water Parameters (Fresh/Start)			Water Parameters (Old/Finish)		
			pH	E.C (µS/cm)	D.O (%)	pH	E.C (µS/cm)	D.O (%)
Clad Gold Mine B	I	0%	6.60	34.7	109.4	6.83	35.5	102.4
		0.03%	6.64	34.0	106.1	6.92	33.9	102.0
		0.1%	6.60	37.6	109.7	6.90	37.3	100.1
		0.3%	6.64	45.4	109.2	6.89	45.2	100.4
		1.0%	6.88	69.6	108.3	7.18	69.5	100.2
		3.0%	6.77	152.3	107.3	7.08	152.4	100.1

NR = Not Reorded

Test Types: H= Feeding
D = Reproduction
I = Acute Immobilisation

Appendix 4: Actual cadmium test concentrations

Test	Type	Treatment	Actual Metal Conc. (µg/L)
Clad -Cd1	Feeding	A	0.08
		B	1.69
		C	2.68
		D	4.50
		E	8.12
		F	13.69
		G	25.84
		Blank	<0.02
Clad-Cd2	Feeding &	A	<0.02
	Reproduction	B	1.41
		C	2.68
		D	4.44
		E	7.95*
		F	14.00
		G	26.99
		Blank	<0.02
Clad-Cd3	Feeding &	A	0.07
	Reproduction	B	1.5
		C	2.77
		D	4.61
		E	8.50
		F	14.63
		G	27.45
		Blank	<0.02

* Concentration determined by linear regression due to an error in chemical analysis

Appendix 4: Actual cadmium test concentrations (continued)

Test Name	Type	Treatment	Actual Metal Conc. (µg/L)
Clad-Cd4	Feeding	A	0.04
		B	1.40
		C	2.88
		D	5.22
		E	8.47
		F	15.80
		G	20.88
		H	28.99
		Blank	<0.02
Clad-Cd5	Feeding	A	0.31
		B	1.81
		C	3.02
		D	4.77
		E	9.25
		F	16.27
		G	28.63
		Blank	<0.02
Clad-Cd6	Feeding	A	0.02
		B	1.5
		C	3.0
		D	5.41
		E	9.50
		F	22.19
		G	27.46
		Blank	<0.02

Appendix 5: Waste water composition

Component (µg/L)	RP2	Gold Mine A	Gold Mine B	Sewage Influent
Ag	-	0.14	8.08	-
Al	138	8099	116.5	41.06
As	-	59.3	15.1	-
Au	-	28.65	65.65	-
B	-	134	141	-
Ba	-	10.7	42.1	-
Be	-	14	<0.5	-
Bi	-	0.14	0.09	-
Br	-	7	41	-
Ca	19	281109	157922	40600
Cd	0.5	26	0.4	0.03
Ce	-	49.35	0.86	-
Co	4	801	694	0.24
Cr	2	2.1	1.4	7.8
Cs	-	35.44	0.4	-
Cu	15	389.9	6409	15.58
Dy	-	5.81	0.09	-
Er	-	2.46	<0.05	-
Eu	-	1.36	<0.05	-
Fe	420	3638	421	166
Ga	-	1.16	0.45	-
Gd	-	7.69	0.11	-
Ge	-	<0.2	<0.2	-
Hf	-	<0.05	<0.05	-
Hg	-	<0.1	<0.1	-
Ho	-	1	<0.05	-
I	-	<0.05	<25	-
In	-	<25	<0.05	-
La	-	21.54	0.54	-
Li	-	263.4	24.7	-
Lu	-	0.21	<0.05	-
Mg (g/L)	131	192	77.13	31.10
Mn	1420	37245	2498.5	22.49
Mo	-	0.52	36.41	-
Nb	-	<0.1	<0.1	-
Nd	-	29.53	0.7	-
Ni	16	556.6	449.40	2.13
Os	-	<0.5	<0.5	-
Pb	4	27.97	1.83	0.3
Pd	-	<0.05	<0.2	-
Pr	-	6.26	0.16	-
Pt	-	<0.05	0.73	-

Appendix 5: Waste water composition (continued)

Component (µg/L)	RP2	Gold Mine A	Gold Mine B	Sewage Influent
Rb	-	135.9	115.8	-
Re	-	0.9	0.3	-
Ru	-	0.15	0.15	-
Sb	-	0.71	1.7	-
Sc	-	7	<2	0.3
Se	4	20	6	-
Sm	-	7.04	0.13	-
Sr	-	260.5	190.2	-
Ta	-	0.23	0.13	-
Tb	-	1.06	<0.05	-
Te	-	<0.5	<0.5	-
Th	-	0.96	0.28	-
Ti	-	28	<10	-
Tl	-	3.6	0.17	-
Tm	-	0.28	<0.05	-
U (g/L)	2237	16.42	0.0009	0.0005
V	-	0.9	<0.2	-
W	-	1.14	4.64	-
Y	-	32.85	0.56	-
Yb	-	1.52	<0.05	-
Zn	39	8760	113.1	13.4
Zr	-	1.3	0.9	-
MBAS	-	-	-	49300
CTAS	-	-	-	200
Diazinon	-	-	-	<1.0
Chloropyriphos	-	-	-	<1.0
Malathion	-	-	-	<1.0
SO4	531000	-	-	14900
Chlorine	-	-	-	20000
Ammonia	-	-	-	44900

Appendix 7: Estimation of algal cell density using a haemocytometer

1. A haemocytometer is a microscope slide containing two chambers each containing a ruled grid shown in Figure A8.1.

2. Moisten the raised polished surfaces of the slide and centre the cover slip. Press down firmly to secure the slip as this should remain attached even if the slide is vigorously shaken.

3. Transfer one drop of the sample onto the slide with a Pasteur pipette. The liquid will be drawn into the chambers by capilliary action.

4. There slide should have a good coverage of solution however it is important to ensure that there is no liquid in the depressions surrounding the chambers and no liquid protruding from the chamber where the sample was loaded.

5. Cells should be allowed to settle for approximately one minute (this time may vary depending on the size of the cells). Both chambers should be counted as soon as possible to avoid drying of the samples.

6. Algal particles are counted in the four outer corners of each grid with cells on the lines of the grid being counted if they are more than halfway over the line into the grid.

7. To calculate cells/mL take the entire count for the two grids and divide by eight and multiply by 10^4.

8. Calculate the number of cells/mL in the diluent water and minus this figure from the sample count to estimate the amount of algae added to the sample.

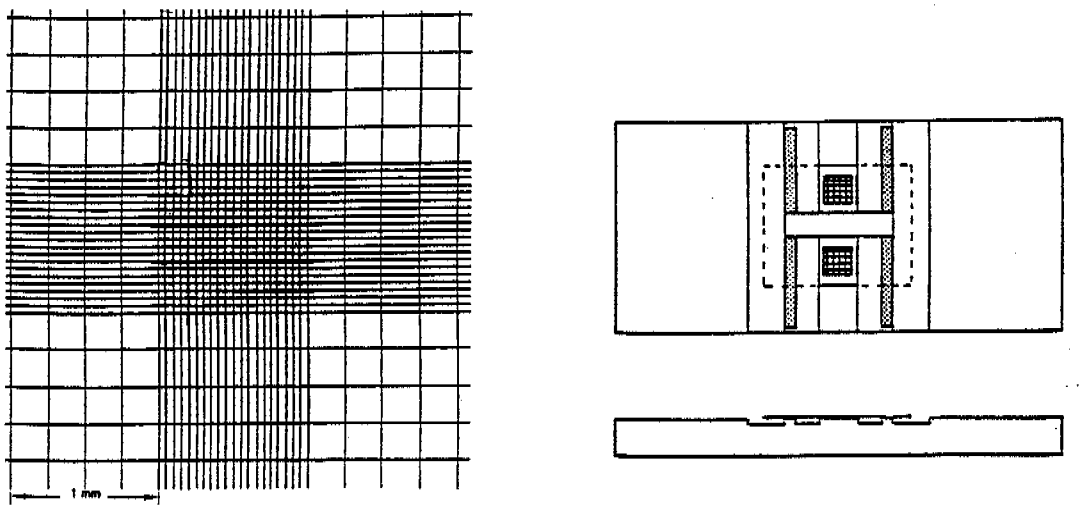

Figure A8.1: Haemocytometer slide and grid

Appendix 8: Preparation of growth media (MBL) for algae

Growth media is prepared monthly for the maintenance of starter cultures, algal slopes and mass culturing of *Chlorella* species. The method for the preparation of MBL is outlined below:

Stock Solutions:

Tris Buffer	100g/L
$NaNO_3$	85.24g/L
$CaCl_2$	27.71g/L
$MgSO_4$	36.97g/L
$NaHCO_3$	12.60g/L
K_2HPO_4	8.72g/L
Na_2EDTA	4.36g/L
$FeCl_3$	0.44g/L
Trace Metals	

Trace Metals (Make up 1L of solution containing the following compounds)

$CoCl_2.6H_2O$	0.010g/L
$CuSO_4$	0.009g/L
$Na2SiO_3$	0.007g/L
$MnCl_2.4H_20$	0.180g/L
$ZnSO_4.7H_2O$	0.022g/L

1. Add 1mL of each of the stock solutions per litre of Milli Q water except Tris Buffer (10mL/L).
2. The pH is ajusted to 7.1-7.3 using 10%HCL or 1M NaOH. 1L and then 1L aliquots are transferred to a 2L conical flasks.
3. The media is autoclaved at 121°C for 20min.

Appendix 9: Percentage of un-ionised ammonia at different pH and temperatures

	pH																			
Temp °C	**6.5**	**6.6**	**6.7**	**6.8**	**6.9**	**7.0**	**7.1**	**7.2**	**7.3**	**7.4**	**7.5**	**7.6**	**7.7**	**7.8**	**7.9**	**8.0**	**8.1**	**8.2**	**8.3**	**8.4**	**8.5**
10	0.0588	0.0740	0.0931	0.117	0.148	0.186	0.234	0.294	0.370	0.465	0.585	0.735	0.924	1.16	1.46	1.83	2.29	2.86	3.58	4.46	5.56
12.5	0.0714	0.0899	0.113	0.142	0.179	0.225	0.284	0.357	0.449	0.564	0.710	0.892	1.12	1.41	1.76	2.21	2.77	3.46	4.31	5.37	6.67
15	0.0865	0.109	0.137	0.172	0.217	0.273	0.343	0.432	0.543	0.683	0.858	1.08	1.35	1.70	2.13	2.66	3.33	4.16	5.18	6.43	7.96
17.5	0.104	0.131	0.165	0.208	0.262	0.329	0.414	0.521	0.655	0.823	1.03	1.30	1.63	2.04	2.56	3.20	3.99	4.97	6.18	7.66	9.45
20	0.125	0.158	0.199	0.250	0.315	0.396	0.498	0.626	0.786	0.988	1.24	1.56	1.95	2.45	3.06	3.82	4.76	5.92	7.34	9.07	11.2
22.5	0.150	0.199	0.238	0.300	0.377	0.474	0.596	0.749	0.942	1.18	1.48	1.86	2.33	2.92	3.65	4.55	5.66	7.02	8.68	10.7	13.1
25	0.180	0.226	0.285	0.358	0.450	0.566	0.712	0.895	1.12	1.41	1.77	2.22	2.78	3.47	4.33	5.39	6.69	8.28	10.2	12.5	15.3
27.5	0.214	0.270	0.339	0.427	0.536	0.674	0.848	1.06	1.34	1.68	2.10	2.63	3.29	4.11	5.12	6.36	7.87	9.72	11.9	14.6	17.7
30	0.255	0.320	0.403	0.507	0.637	0.801	1.01	1.26	1.58	1.99	2.49	3.11	3.89	4.85	6.02	7.47	9.22	11.3	13.9	16.9	20.3

Illustration on how to calculate concentration of total ammonia-N (as µg/L):

For a solution with un-ionised NH_3 of 68.7 µg/L at pH 7 and temperature 25°C, the concentration of total ammonia-N is:

Total ammonia-N (µg/L) = un-ionised ammonia as µg NH_3/L X (14/17) / (% un-ionised ammonia/100)

Source: ANZECC, (2000) Australian and New Zealand Guidelines for Fresh and Marine Water Quality – Volume 2, Aquatic Ecosystems Rationale and Background Information. Australia and New Zealand Environment and Conservation Council and Agriculture and Resource Management Council of Australia and New Zealand.