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The effect of true water hardness and alkalinity on the toxicity of Cu and U to two tropical Australian freshwater organisms

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List of abbreviations

ANZECC	Australian and New Zealand Environment and Conservation Council
ARMCANZ	Agriculture and Resource Management Council of Australia and New Zealand
ARR	Alligator Rivers Region
BEC_{10}	10% Bounded-effect-concentration
CCREM	Canadian Water Quality Guidelines
C.I.	Confidence Interval
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EC_{50}	50% Effect-Concentration
eriss	Environmental Research Institute of the Supervising Scientist
FIAM	Free-Ion Activity Model
HARPHRQ	HARwell PH and Redox eQuilibrium
LC_{50}	Median-Lethal-Concentration
LOEC	Lowest-Observed-Effect-Concentration
MDEC	Minimum-Detectable-Effect-Concentration
MES	2-Morpholinoethanesulfonic acid
NOEC	No-Observed-Effect-Concentration
WQGs	Water Quality Guidelines

List of publications

Refereed publications

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Abstract

The Australian and New Zealand water quality guidelines aim to supplement and modify existing criteria, which are mostly based on Northern Hemisphere toxicity data, with information relevant to Southern Hemisphere ecosystems as it becomes available. In the wet-dry tropics of Australia, copper (Cu) and uranium (U) are metals of particular concern, due to mining activities. Although, the toxicity of Cu and U to tropical freshwater species has previously been characterised, the influence of physico-chemical parameters on toxicity has not been defined. In contrast, temperate freshwater studies have investigated the effects of various physico-chemical parameters on Cu toxicity, and to a limited extent U toxicity. The reported results however, are contradictory. Thus, it is recognised that the development of a model based on key water quality variables would enhance the capacity to predict the potential site-specific impacts of Cu and U in tropical ecosystems.

This study aimed to separate the effects of true water hardness (6.6, 165 and 330 mg L^{-1} as $CaCO_3$) and alkalinity (4.0 and 102 mg L^{-1} as $CaCO_3$), at constant pH, on the toxicity of Cu and U to *Hydra viridissima* (Green hydra, population growth) and *Mogurnda mogurnda* (Purple-spotted gudgeon, sac-fry survival). The effect of water hardness (ie. Ca and Mg concentration) varied depending on the metal and test organism. A 50-fold increase in hardness resulted in a 2-fold decrease in the toxicity of Cu to *M. mogurnda*, while it had no effect on U toxicity. The opposite was observed for *H. viridissima*, where increased hardness had no effect on Cu toxicity, but decreased U toxicity by approximately 2-fold. A 25-fold increase in alkalinity (ie. carbonate concentration) had no effect on Cu toxicity to *H. viridissima*, while it decreased U toxicity by approximately 10%. Gaining a fundamental understanding of the interactions between physico-chemical parameters and metals, and the subsequent potential impacts on freshwater ecosystems is an essential aspect of site-specific environmental risk assessment and water quality guideline derivation.

1 Introduction

1.1 Background

Metal contamination from mining and industrial activities is an increasing threat to aquatic ecosystems. The presence of metals in the environment increases either directly via atmospheric deposition, wastewater discharge and runoff (eg. Pb, Hg, Cd, Cu and Zn), or indirectly as a result of increased solubilisation and mobilization from sediments (eg. Al and Fe). While both marine and freshwater ecosystems are threatened, soft freshwaters are particularly sensitive, as they are poorly buffered and prone to acidification (McDonald and Wood, 1993). In addition, metal speciation and bioavailability in fresh surface waters are known to depend on a variety of physico-chemical parameters (eg. temperature, dissolved organic carbon (DOC), pH, hardness and alkalinity). For this reason, the ability to protect aquatic ecosystems from metal contamination depends on understanding tolerance limits of aquatic biota, and the factors influencing these limits. Safe metal concentrations are recommended by the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ, 1999) to protect aquatic ecosystems.

The metals which are of greatest concern to tropical Australian freshwaters are Al, Cd, Co, Cu, Ni, Mn, Pb, U, V and Zn, largely a consequence of mining activities, but also from urban impacts (Refer to review by Markich and Camilleri, 1997). Copper and U were selected for this study because their toxicity to tropical biota has been comprehensively described. However, high variability in the toxic response of these two metals in tropical freshwaters remains (Appendix A). Markich and Camilleri (1997) proposed such variability may be reduced by elucidating the effect physico-chemical parameters (eg. hardness, alkalinity, pH, natural organic matter and redox potential) have on the toxicity of these two metals to aquatic biota. Knowledge of the relationship between water chemistry variables, including hardness and alkalinity, and metal toxicity is useful for predicting the potential ecological detriment to aquatic systems, and can be used to modify national water quality guidelines on a site-specific basis.

1.1.1 Significance of water quality guidelines

Water quality guidelines (WQGs) provide a means of assessing the 'water quality' required to protect aquatic ecosystems at a prescribed level (Chapman, 1995). Currently, Australian and New Zealand aquatic biota are protected by guidelines based predominately on toxicity data for Northern Hemisphere species (ANZECC, 1992). However, this has been necessary, as local toxicological data relevant for Australian species are limited or lacking. The ability of Northern Hemisphere data to reflect Australian climatic and limnologic conditions, as

well as the phylogeny of species, has often been questioned (Skidmore and Firth, 1983; Markich and Camilleri, 1997). For example, tropical Australian freshwater systems experience seasonal variations in water quality parameters, such as low conductivity or hardness during the Wet season and high temperature or low dissolved oxygen during the Dry season, which are beyond ranges so far studied in the Northern Hemisphere (Skidmore and Firth, 1983). In addition, the majority of Australia's freshwater fish and invertebrates are endemic, with none of the species used in Northern Hemisphere toxicity tests (predominantly Salmonidae and Cyprinidae) occurring naturally in Australia (Skidmore and Firth, 1983). The inability of the current Australian WQGs (ANZECC, 1992) to reflect such environmental differences, casts doubt over their level of protection. Of particular concern, is the relevance of temperate based guidelines for protecting tropical Australian biota, given that tropical Australia encompasses 46% of the Australian continent (ASTEC, 1993).

1.1.2 Water quality guidelines relevant to tropical Australia

The aim of the Australian and New Zealand WQGs is "to protect all forms of aquatic life cycle ... during indefinite exposure to the water" (ANZECC, 1992). The achievement of such an aim is dependent on the quality of toxicological data for Australian and New Zealand biota, and the ability to predict potential impacts on biota under site-specific conditions. The potential impact of mine wastewater and its constituents is just one environmental issue tropical freshwater systems face (Markich and Camilleri, 1997). Of particular interest to the present study, is the presence of Cu and U in mine wastewaters and the potential environmental impact such metals have to aquatic organisms inhabiting the Wet/Dry tropics. Only in the recent draft of the Australian and New Zealand water quality guidelines, was a guideline for U recommended to be 39 $\mu g\ L^{-1}$ (ANZECC and ARMCANZ, 1999). However, there is no provision in the guidelines to use an algorithm to modify the guideline value to account for different hardness.

A recent review of available metal toxicity data to aquatic biota in tropical Australia (Markich and Camilleri, 1997), highlighted the need to better describe the tolerance limits of aquatic biota to metals, and the factors influencing these limits. Physico-chemical parameters such as water hardness, alkalinity, pH and dissolved organic carbon (DOC) are factors known to potentially modify metal toxicity and bioavailability (Hamelink *et al.*, 1994; Markich *et al.*, 1997). Quantitative relationships (algorithms) describing the reduction in bioavailability as a function of increasing water hardness have been established for Cd, Cr(III), Cu, Ni, Pb and Zn. However, water hardness has yet to be quantified and incorporated into the existing water quality guidelines for other metals (eg. U). It is recognised that the development of a model based on key water quality variables would

enhance the capacity to predict the potential site-specific impacts of U and Cu in tropical aquatic ecosystems.

1.1.3 Importance of physico-chemical parameters in determining metal toxicity

Historically, water quality guidelines have been based on the 'total' aqueous concentration of a metal. However, evidence has established that the 'bioavailable' metal concentration (ie. the potential of a metal to enter and interact physiologically with a living system) more accurately predicts the toxic effects of metals (Campbell, 1985; Markich, 1998). Apart from the potential uptake of the metal, the toxicity of the metal also depends on the form and abundance in which a particular species of metal is present. Metals which are present as the free ion, or as a weak metal complex are more bioavailable than metals in strong complexes or adsorbed to colloidal and/or particulate matter (Markich *et al.*, 1997). Physico-chemical variables, such as hardness, pH, alkalinity and DOC may influence the speciation and bioavailability of metals (Hamelink *et al.*, 1994; Markich *et al.*, 1997). Determining the influence of inorganic complexation is difficult, as the effects of pH, alkalinity and hardness are often difficult to separate. An increase in water hardness is frequently associated with an increase in alkalinity (where Ca and/or Mg are added as carbonate), and often pH. Alkalinity and pH influence metal speciation by changing the free carbonate and hydroxide ion concentration, whereas hardness (Ca and/or Mg concentration) typically has no direct effect on metal speciation in solution, and only minor indirect effects via changes in ionic strength (Hunt, 1987). Calcium and Mg do, however, seem to affect cell membrane permeability and/or compete with trace metals for transport uptake sites (Hunt, 1987; Markich and Jeffree, 1994).

The current draft of the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ 1999) recognize the potential influence of varying hardness on Cd, Cr(III) Cu, Pb, Ni and Zn toxicity in freshwaters. Subsequently, a quantitative method to calculate a metal guideline value with respect to a particular water hardness level has been provided. However, for these guideline values to comprehensively protect aquatic biota, such algorithms need to be derived for other priority metals (eg. U).

1.2 Copper

1.2.1 Significance of Cu in tropical Australian freshwaters

For most aquatic organisms, Cu is essential in trace amounts, but may be one of the most toxic metals when natural concentrations are elevated (Skidmore and Firth, 1983; Nor, 1987). The current water quality guidelines for the protection of aquatic ecosystems recommend a total Cu concentration of 2-5 $\mu g\ L^{-1}$, depending on water hardness (ANZECC,

1992). However, no quantitative formula is provided in the guidelines, which specifies a particular Cu concentration for a particular hardness.

1.2.2 Chemistry and speciation of Cu in natural waters

In fresh surface waters, Cu may exist in hydrated ionic forms, complexes and/or sorbed to a variety of naturally occurring organic and inorganic compounds (Table 1.1) (Leckie and Davis, 1979; Flemming and Trevors, 1989). The free cupric ion (Cu^{2+}) is considered the most predominant form (at pH <6.5), and the most toxic to aquatic organisms (Markich *et al.*, 1997). The concentration of Cu^{2+} is controlled by physico-chemical variables including pH, organic ligands/agents (eg. humic and fulvic acid), and inorganic ligands (eg. phosphates and carbonates) (Markich *et al.*, 1997).

Table 1.1: Physico-chemical forms of Cu in natural waters[a].

Physico-chemical form	General size and form	Example
Simple ionic species	true solution (< 0.001 µm)	$Cu(H_2O)_6^{2+}$
Weak complexes	"	Cu-fulvic acid
Lipid-soluble complexes	"	Cu-oxinate
Organo-metallic species	"	Cu-citrate
Adsorbed on colloid particles	colloid (0.001-0.1 µm)	Cu-$Fe(OH)_3$-humic acid
Adsorbed on particles	particulate (0.1-50 µm)	Cu adsorbed onto or contained within clay particles

[a] Modified from Florence and Batley (1980).

The dominance of the free cupric ion (Cu^{2+}) at pH ≤ 6.0 is offset by the abundance of cupric-carbonate and -hydroxy species at pH ≥ 7.0 (Apte and Day, 1993). Above pH 6.0, the concentration of Cu^{2+} declines by an order of magnitude for each 0.5 units increase in pH (Stumm and Morgan, 1981). Of the Cu hydroxy species, $CuOH^+$ increases in importance over the pH range 6-8, and $Cu(OH)_2$ (aq) increases in importance over the pH range 8-11, while both species are equivalent at approximately pH 8 (Markich *et al.*, 1997). The percentage of $CuCO_3$ also increases in abundance as the Cu^{2+} declines from pH 6 to 8 (Sylva, 1976), where the abundance of $CuCO_3$ increases with increasing alkalinity, peaking around pH 8 (Miwa *et al.*, 1989). The complexation of Cu by sulfate, chloride, nitrate and phosphate depends on the concentration of individual anions, but generally these complexes comprise less than 5% of dissolved Cu in freshwaters (French, 1986).

Copper(II) readily complexes with natural dissolved organic matter (DOM), forming strong bonds with ligands containing oxygen, nitrogen and sulfur (Hart, 1981; Moore and Ramamoorthy, 1984). Subsequently, the majority of Cu in natural waters (90-100%) is present as Cu-DOM complexes, while inorganic Cu species represent a relatively small proportion of the total dissolved Cu (Apte and Day, 1993). The percentage of Cu-DOM complexes in freshwaters will increase as the pH and DOM concentration increase, and the concentration of competing ions decrease (Sylva, 1976).

The fate of Cu^{2+} in aquatic systems is strongly influenced by sorption in the presence of Fe, Al and Mn (oxy)hydroxides, clay and carbonate minerals, insoluble organic matter and biotic surfaces (Leckie and Davis, 1979; Dzombak and Morel, 1990). Sorption of Cu^{2+} to oxyhydroxides increases with pH up to a threshold point, which is dependent on the concentration of Cu, adsorbent, competing ions and ionic strength (Dzombak and Morel, 1990). The majority of Cu^{2+} is sorbed around pH 7 in most fresh surface waters (Moore and Ramamoorthy, 1984; Dzombak and Morel, 1990).

1.2.3 Toxicity of Cu to tropical Australian freshwater species

The toxicity of Cu to organisms from several phyla, including Chordata (Osteichthyes), Mollusca, Cnidaria, Crustacea and Chlorophyta (Appendix A), inhabiting tropical Australian freshwaters has been determined. A recent review by Markich and Camilleri (1997) details this information. Only those studies, which have investigated the toxicity of Cu to Hydra species and Purple-spotted gudgeon (*Mogurnda mogurnda*), are discussed here.

Hydra

The relative sensitivity of freshwater hydra to Cu between studies is difficult to compare (Table 1.2), as authors have used different methodologies. Markich and Camilleri (1997) assessed the toxicity of Cu to the green hydra, *Hydra viridissima*, in a reconstituted soft freshwater (pH 6.0 ± 0.1; hardness 3.9 mg L^{-1} as $CaCO_3$). Population growth was reduced by 50% (ie. Effect concentration; EC_{50}) at 4 µg L^{-1} Cu, while the 10% bounded effect concentration (BEC_{10}), an alternative estimate to the no-observed-effect concentration (NOEC), was 1.6 µg L^{-1} Cu (Markich and Camilleri, 1997). Pollino and Holdway (1999) found *H. viridissima* to be less sensitive to Cu in a laboratory water at pH 7.2 ± 0.4 (hardness 20 mg L^{-1} $CaCO_3$, and an unknown organic composition). In their study, the 96 h LC_{50} (median lethal concentration) and NOEC were calculated to be 8.5 ± 0.3 and 4.0 µg L^{-1} Cu, respectively.

Hydra viridissima was found to be three times more sensitive to Cu than the pink hydra, *Hydra vulgaris*, with the 96 h LC_{50} values being 8.5 µg L^{-1} and 26 µg L^{-1}, respectively (Pollino and Holdway, 1999). Allison and Holdway (1988) also reported *H. viridissima* to

be a more sensitive species than *H. vulgaris* to U (Table 1.3). Beach and Pascoe (1998) reported the 48 h and 96 h LC_{50} of Cu to *H. vulgaris* to be 190 and 40 μg L^{-1} Cu, respectively, while a 50% reduction in feeding rate was observed at 10 μg L^{-1} Cu. The median lethal concentration may be an important value, however, the substantially lower Cu concentration required to reduce feeding rate, compared to that causing 50% mortality, also has important behavioural implications in assessing environmental impacts.

Stebbing and Pomroy (1978) investigated the response of a temperate hydra species, *Hydra littoralis* to Cu. The rate of asexual reproduction was significantly ($P \leq 0.05$) inhibited by 4.0 μg L^{-1} Cu. It is difficult to compare the response of *H. littoralis* to the tropical species, *H. viridissima* and *H. vulgaris*, due to differences in experimental conditions such as test endpoint and physico-chemical parameters of the test waters (Table 1.2). Stebbing and Pomroy (1978) reported a linear relationship between metal levels accumulated in hydra tissue and nominal metal exposure levels. This supports the finding that *Hydra sp.*, like other aquatic invertebrates, are unable to regulate the uptake of Cu (Bryan, 1976; Hyne *et al.*, 1993).

Purple-spotted gudgeon (M. mogurnda)

The sensitivity of *M. mogurnda* to Cu appears to differ between natural water and synthetic water (inorganic component of natural water) (Table 1.2). This is not surprising considering Cu toxicity is known to decrease in the presence of organic matter (Breault et al, 1996). In natural Magela Creek (Buffalo Billabong) water, Rippon and Hyne (1992) found a 96 h NOEC of 20 μg L^{-1} Cu and a 96 h lowest observed effect concentration (LOEC) of 64 μg L^{-1} Cu on *M. mogurnda* sac-fry survival. Markich and Camilleri (1997) reported *M. mogurnda* to be two-fold more sensitive in synthetic water than natural water, having a BEC_{10} of 12 μg L^{-1} and a minimum detectable effect concentration (MDEC; analogous to LOEC) of 13 μg L^{-1}. Such a difference between studies could be explained by the reduction in bioavailable Cu concentration in the natural water as a result of Cu-organic complexation. However, Rippon and Hyne (1992) did not measure the dissolved organic carbon (DOC) concentration in their test water. The sensitivity of *M. mogurnda* to Cu has not been directly compared with a range of other species in a single study, however, *M. mogurnda* appears to be among the more sensitive fish species to metals compared to those investigated in independent studies (Appendix A). This supports the findings of Bywater *et al.* (1991) for U sensitivity.

Table 1.2: Summary of Cu toxicity data for Hydra and Purple-spotted gudgeon[a].

Species	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Test Endpoint	Water Concentration (µg Cu L^{-1})	Reference
Green hydra *(Hydra littoralis)*	Lomis, 1954 Synthetic medium	NR[h]	NR[h]	NR[h]	264 h (11 d) mean rate of reproduction	4.0 (LOEC)[f]	Stebbing & Pomroy (1978)
Green hydra *(Hydra viridissima)*	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	96 h population growth	1.6 (BEC_{10})[b] 1.8 (MDEC)[c] 4.0 (EC_{50})[d] (3.8-4.2)	Markich & Camilleri (1997)
	Autoclaved mains	7.2 ± 0.4	20	NR[h]	96 h population growth	4 (NOEC)[e] 8 (LOEC)[f] 8.5 (LC_{50})[g]	Pollino & Holdway (1999)
Pink hydra *(Hydra vulgaris)*	Autoclaved mains	7.2 ± 0.4	20	NR[h]	96 h population growth	4 (NOEC)[e] 8 (LOEC)[f] 26 (LC_{50})[g]	Pollino & Holdway (1999)
	Lenhoff, 1983 M Solution	7.8	NR[h]	NR[h]	24h population growth	410 (LC_{50})[g]	Beach & Pascoe (1998)
					48 h population growth	190 (LC_{50})[g]	
					48 h feeding inhibition	10 (EC_{50})[d]	
					96 h population growth	40 (LC_{50})[g]	
Purple-spotted gudgeon *(Mogurnda mogurnda)*	Buffalo Billabong	6.5	4 (3-5)	3 (2-4)	96 h sac-fry survival	20 (NOEC)[e] 64 (LOEC)[f]	Rippon & Hyne (1992)
					120 h embyro hatching	> 200 (LOEC)[f]	
	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	96 h survival	12 (BEC_{10})[b] 13 (MDEC)[c] 23 (LC_{50})[g] (22-24)	Markich & Camilleri (1997)

Table 1.2 Cont'd

[a] All numerical values represent mean values, or their range, with 95% confidence intervals (C.I) in parentheses (where reported). Means shown with ± values were regulated within the reported limits. Uranium (U) concentration is expressed as uranyl (ie. UO_2); this was derived by multiplying the U concentration by 1.14.

[b] BEC_{10}, 10% bounded-effect concentration (Hoekstra and van Ewijk, 1993), an analogous statistical measure of the no-observed effect concentration (NOEC).

[c] MDEC, minimal detectable effect concentration (Ahsanullah and Williams, 1991), an analogous statistical measure of the lowest-observed effect concentration (LOEC).

[d] EC_{50}, median effect concentration.

[e] NOEC, no-observed effect concentration.

[f] LOEC, lowest-observed effect concentration.

[g] LC_{50}, concentration at which there is 50% survival.

[h] Not Reported.

1.2.4 Mechanisms of Cu toxicity in water

Upon diffusion of a metal through the protective layer of a living organism, the incoming metal will encounter a range of potential binding sites (Campbell, 1985). The metal may 'collect' without affecting normal cell function or be taken up, perturbing processes such as photosynthesis, respiration, motility, growth and reproduction.

Copper is considered highly toxic to Hydra, particularly green hydra (*H. viridissima*). The symbiotic algae hosted by *H. viridissima* help regulate exposure to elevated levels of a metal by accumulating excess metal and being shed from the host tissue (Hyne *et al.*, 1993). However, this mechanism may be inadequate in the presence of Cu due to Cu being such a potent algicide (Pollino and Holdway, 1999). Copper has been found to inhibit the photosynthesis of an Australian tropical *Chlorella sp.* at 1.6 $\mu g\ L^{-1}$ Cu (Franklin *et al.*, 1998). At non-toxic concentrations, Cu has been reported to increase hydra population growth (Stebbing and Pomroy, 1978; Pollino and Holdway, 1999). Hormesis was found to occur at Cu concentrations below 5 $\mu g\ L^{-1}$ for *H. littoralis* (Stebbing and Pomroy, 1978) and below 8 $\mu g\ L^{-1}$ for *H. vulgaris* and *H. viridissima* (Pollino and Holdway, 1999).

Gill surfaces of fish have been identified as the primary uptake site of several waterborne metals (Cu^{2+}, Laurén and McDonald, 1986; Reid and McDonald, 1991; Cd^{2+}, Part *et al.*, 1985; Reid and McDonald, 1988; Zn^{2+}, Hogstrand *et al.*, 1994; Al^{3+}, Verbost *et al.*, 1992). The permeability of the gill surface is expected to be greater if the membrane has a low affinity for the metal (Reid and McDonald, 1991). Once through the membrane and in the intracellular compartment the metal is exposed to various complexing ligands. Metals may bind to these ligands, resulting in one or more of the following mechanisms of toxicity: a) Blocking of essential biological functional groups in biomolecules; b) Displacement of essential metal ions in molecules; and c) Modification of active conformation of biomolecules (Reid and McDonald, 1991). These mechanisms can be used to describe osmoregulation inhibition by Cu^{2+} exposure (Laurén and McDonald, 1986; Reid and McDonald, 1988). Copper(II) has been found to disrupt gill functioning by forming covalent bonds with nitrogen/sulphur-rich centers such as those of APTase (Reid and McDonald, 1988).

Surface bound Ca^{2+} is known to stabilize the gill membrane, consequently reducing ionic permeability (Flik and Verbost, 1994). It is hypothesized that increased Ca^{2+} concentrations in solution further protect aquatic biota from toxic trace metals by competing with the free ionic species for binding sites at the gill surface (Markich and Jeffree, 1994).

The H^+ ion has been found to disrupt gill functioning in rainbow trout (*Salmo gairdneri*; renamed *Oncorhynchus mykiss*) by impairing transepithelial ion exchange (Reid and McDonald, 1988). The mechanism by which H^+ affects gill permeability may be related to its charge, ionic radius, ligand binding preference (eg. oxygen versus nitrogen or sulphur centers), and binding affinity (Reid and McDonald, 1991).

1.2.5 Effect of physico-chemical parameters on Cu toxicity

It is generally accepted that increased water hardness reduces the toxicity of Cu to freshwater organisms (refer to reviews by Sorensen, 1991 and Mayer *et al.*, 1994). Conversely, Winner (1985) and Laurén and McDonald (1986) found increasing hardness had little or no effect on the toxicity of Cu. Several studies (Howarth and Sprague, 1978; Gauss *et al.*, 1985; Belanger *et al.*, 1989) which provide evidence in support of the inverse relationship between hardness and Cu toxicity, confounded the effects of true water hardness (ie. Ca and/or Mg concentrations) by accompanying changes in hardness with changes in alkalinity and pH. For example, Howarth and Sprague (1978) reported the 96 h LC_{50}, for rainbow trout (*Salmo gairdneri*) exposed to Cu, to vary from 20 $\mu g\ L^{-1}$ in soft acid water, to 520 $\mu g\ L^{-1}$ in hard alkaline water, where hardness ranged from 30 to 360 $mg\ L^{-1}$ as $CaCO_3$, and pH from 5 to 9. In many freshwater systems hardness has a strong positive correlation with alkalinity and pH, however, confounding the effects of these physico-chemical parameters has important implications if the effects of 'true water hardness' on Cu toxicity are assumed to be constant over an infinitely wide range of water qualities.

Several studies have identified the need to discern the effects of true water hardness (ie. Ca and/or Mg concentration) on copper-organism interactions, and have successfully described the relationship by maintaining constant alkalinity and pH. Increasing water hardness was found to ameliorate the toxicity and bioavailability of Cu to aquatic biota (Miller and Mackay, 1980; Mierle, 1981; Horne and Dunson, 1995 and Erickson *et al.*, 1996). The toxicity of Cu is reduced by the Ca^{2+} and/or Mg^{2+} ions competing with Cu^{2+} ions for binding sites at the cell surface of organisms, without directly effecting Cu speciation (Markich and Jeffree, 1994; Erickson *et al.*, 1996). More specifically, Ca has been identified as having a greater inhibitory effect than Mg, on the toxicity of Cu to aquatic organisms (O'Shea and Mancy, 1978; Erickson *et al.*, 1996).

The effect of increasing alkalinity (log pCO_2) has also been reported to reduce the toxicity and bioavailability of Cu to freshwater biota, in experiments that manipulated the carbonate concentration independently of the Ca and Mg concentration, and pH (Andrew *et al.*, 1977; Miller and Mackay, 1980; Laurén and McDonald, 1986; Daly *et al.*, 1990b). Alkalinity may have reduced Cu toxicity via the formation of Cu-carbonate complexes, which decrease the activity of the free metal ion (Cu^{2+}) (Borgmann, 1983; Hunt, 1987).

Solution pH is a primary variable influencing the toxicity of metals, yet the literature describes opposing effects of pH. Many studies have found Cu to be less toxic with freshwater acidification, over a pH range of 3.0 to 7.0 (Campbell and Stokes, 1985; Cusimano *et al.*, 1986; Macfie *et al.*, 1994; Horne and Dunson, 1995; Franklin *et al.*, 1998). The protective effect of low pH on Cu toxicity is considered a function of H^+ competitively inhibiting Cu^{2+} at metal transport sites on the cell membrane (Pagenkopf, 1983; Campbell and Stokes, 1985; Gerhardt, 1993). In contrast, some studies have reported an increase in Cu toxicity with a reduction in pH, over the pH range 6.0 to 8.5 (Waiwood and Beamish, 1978; Schubauer-Berigan *et al.*, 1993; Erickson *et al.*, 1996).

Copper toxicity has been reported to decrease in the presence of organic complexing agents (Meador, 1991; Welsh *et al.*, 1993; Azenha *et al.*, 1995; Erickson *et al.*, 1996, Hansten *et al.*, 1996), while other studies suggest that under certain conditions Cu toxicity may be enhanced (Guy and Kean, 1980; Daly *et al.*, 1990a; Tubbing *et al.*, 1994; Buchwalter *et al.*, 1996). The attenuating effects of natural DOC (eg. humic and fulvic acids) and synthetic organic agents (eg. EDTA) in surface waters (pH 5-9), is attributed to their ability to complex with Cu. In contrast, Cu-organic complexes may increase Cu toxicity by facilitating the transport of cupric ions into cells and/or increasing cell permeability (Guy and Kean, 1980; Daly *et al.*, 1990a). Further studies are required to validate such relationships.

1.3 Uranium

1.3.1 Significance of U in tropical Australian freshwaters

The surface waters of rivers and streams in tropical Australia, particularly the Northern Territory, typically contain less than 1 $\mu g\ L^{-1}$ U (Hart *et al.*, 1987; Markich, 1998). Uranium is non-essential for biological processes and is generally toxic at elevated concentrations (Berlin and Rudell, 1979). Since U is highly soluble and mobile in natural waters (Morse and Choppin, 1991), contaminated waters from local mining activities are a potential hazard to aquatic biota. Given the presence of U mines in tropical Australia, the toxicity of U to freshwater biota has been frequently studied (Bywater et al., 1991; Holdway, 1992; Markich and Camilleri, 1997; Markich, 1998).

1.3.2 Chemistry and speciation of U in natural waters

In aquatic environments, U may exist in many soluble forms, including the dissolved uranyl ion (UO_2^{2+}) and other uranyl complexes such as $(UO_2)_3(OH)_5^-$, $UO_2(CO_3)_2^{2-}$ and $(UO_2(HPO_4)_2)^{2-}$ (Langmuir, 1978; Markich *et al.*, 1996). There is considerable evidence suggesting the hexavalent uranyl ion (UO_2^{2+}) predominates in oxidized surface waters and forms stable, readily soluble, cationic, anionic and/or neutral complexes which are highly mobile (Langmuir, 1978; Osmond and Ivanovich, 1992; Markich *et al.*, 1996). Suspended

particles, pH, redox potential, organic complexes and inorganic ligands (such as phosphates and carbonates) govern the speciation of U and its abundance in natural waters.

The speciation of U is highly pH-dependant. At pH ≤ 5.0, the free hydrated uranyl ion (UO_2^{2+}) predominates, becoming insignificant at pH ≥ 6.0, in waters containing environmentally relevant concentrations of dissolved U (< 10 µg L^{-1}) (Grenthe *et al.*, 1992; Markich *et al.*, 1996). The second most dominant species at pH 5 is UO_2OH^+, which increases in importance up to pH 6 (Grenthe *et al.*, 1992; Markich *et al.*, 1996). The formation of polymeric uranyl-hydroxide complexes including $(UO_2)_2(OH)_2^{2+}$, $(UO_2)_3(OH)_5^+$, $(UO_2)_4(OH)_7^+$ and $(UO_2)_3(OH)_7^-$ increase in importance at pH ≥ 5.0, particularly at higher U concentrations (Grenthe *et al.*, 1992; Markich *et al.*, 1996). Markich (1998) provided evidence to suggest that UO_2^{2+}, and to a lesser extent UO_2OH^+, are the U species which contribute most to the toxic response observed in aquatic biota, where UO_2^{2+} has approximately twice the effect of UO_2OH^+.

Carbonate is considered the most significant inorganic complexing agent of uranyl ions due to the formation of very stable complexes (Greene *et al.*, 1986). In moderate to hard waters (ie. hardness and alkalinity > 60 mg L^{-1} $CaCO_3$) at pH 5-6, UO_2CO_3 is the dominate species, while at pH 6-8, $UO_2(CO_3)_3^{4-}$ is the dominant species. The complexation of uranyl by chloride, sulfate, nitrate, and silicate is considered relatively weak compared to uranyl complexes with carbonate and phosphate in freshwaters (Gascoyne, 1992). Uranyl-phosphate complexes only start to become significant when the concentration of phosphate approaches 75 µg/L (Langmuir, 1978).

Dissolved organic matter, as humic and fulvic acids, is known to form stable complexes with uranyl ions in natural waters (Choppin, 1992; Markich, 1998). Soluble uranyl-DOM complexes contribute to the migration of uranyl ions in water (Moulin *et al.*, 1992), while insoluble uranyl-DOM complexes may reduce the bioavailability and toxicity of U to aquatic organisms by acting as a sink for U (Brown *et al.*, 1994). In organic-rich freshwaters which have a low hardness and alkalinity (pH 5-7), the uranyl-DOM complexes are considered the dominant species of dissolved U (Markich, 1998). However, as the hardness, alkalinity and pH (usually pH >7-8) of the water increases there is a shift in speciation, where uranyl-carbonate and uranyl-hydroxide-carbonate species become more important than uranyl-DOM complexes (Moulin *et al.*, 1992).

The fate of U in freshwaters is also known to be significantly influenced by sorption to clay minerals below pH 5, and Fe and Al (oxy)hydroxides, silica and microorganisms at higher pH (Greene *et al.*, 1986; McKinley *et al.*, 1995; Kohler *et al.*, 1996). Sorption of U to particles is typically elevated with increasing pH up to a threshold point, which depends on

the concentration of U, adsorbent, competing ions (eg. carbonate), chelating agents and ionic strength (Markich *et al.*, 1997). In fresh surface waters (pH 6-8), the solubility of uranyl minerals is close to minimum (Langmuir, 1978), while the sorption of uranyl by organic matter is close to maximum (Choppin, 1992).

1.3.3 Toxicity of U to tropical Australian freshwater species

The toxicity of U to organisms from several phyla, including Chordata (Osteichthyes), Mollusca, Cnidaria, Crustacea and Chlorophyta (Appendix A), inhabiting tropical Australian freshwaters has been determined. This database contains an extensive summary of U ecotoxicological information, which is non-existent for other tropical continents. A recent review by Markich and Camilleri (1997) detailed this information. Only those studies, which have investigated the toxicity of U to Hydra species and Purple-spotted gudgeon (*M. mogurnda*), are discussed here.

Hydra

The toxicity of U to hydra has been reported in three studies (Table 1.3). Allison and Holdway (1988) investigated the effects of U toxicity to population growth of green hydra, *H. viridissima* and pink hydra, *H. vulgaris*. *H. viridissima* was found to be approximately five-fold more sensitive to U than the pink hydra, *H. vulgaris* (Table 1.3). Investigations using natural Magela Creek (Buffalo Billabong) water found U concentrations $\geq$ 160 $\mu g\ L^{-1}$ inhibit *H. viridissima* population growth (Allison and Holdway, 1988). However, in synthetic Magela Creek water, *H. viridissima* was three times more sensitive to U with an EC_{50} of 108 $\mu g\ L^{-1}$ (Markich and Camilleri, 1997). Despite the ionic composition of the synthetic water mimicking the natural creek water, the slightly greater pH (~0.5 units) and un-reported DOC content of the natural water probably contribute to the apparent difference.

Purple-spotted gudgeon (M. mogurnda)

Bywater *et al.* (1991) compared the relative sensitivity of six fish species at various life stages to U in natural Magela Creek water over a 96 h period, to establish the most suitable species to assess the toxicity of U mine wastewater. *Mogurnda mogurnda* was found to be the third most sensitive species: being less sensitive than Delicate blue-eye (*Pseudomugil tenellus*) and Reticulated perchlet (*Ambassis macleayi*); and more sensitive than Mariana's hardyhead (*Craterocephalus marianae*), Black-striped rainbowfish (*Melanotaenia nigrans*) and Chequered rainbowfish (*Melanotaenia splendida*). Based on the sensitivity to U, any of these species would be suitable to assess U mine wastewater. However, *M. mogurnda* proved to be the most acceptable, as the larval stages can be easily fed and produced in numbers sufficient for laboratory bioassays.

Table 1.3: Summary of U toxicity data for hydra species and Purple-spotted Gudgeon[a].

Species	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Test Endpoint	Water Concentration (µg U L^{-1})	Reference
Green hydra (*Hydra viridissima*)	Buffalo Billabong	6.5 ± 0.2	4 (3-5)	3 (2-4)	96 h population growth	160 (LOEC)[f] (Dry season) 194 (LOEC)[f] (Wet season)	Allison & Holdway (1988)
	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	96 h population growth	56 (BEC_{10})[b] 61 (MDEC)[b] 108 (EC_{50})[b] (102-114)	Markich & Camilleri (1997)
Pink hydra (*Hydra vulgaris*)	Buffalo Billabong	6.4 ± 0.1	4 (3-5)	3 (2-4)	96 h population growth	740 (LOEC)[f] (Dry season)	Allison & Holdway (1988)
Purple-spotted gudgeon (*Mogurnda mogurnda*)	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	48 h survival	2340 (LC_{50})[g] (1860-2790)	Bywater *et al.* (1991)
					72 h survival	1265 (LC_{50})[g] (950-1650)	
					92 h survival	1265 (LC_{50})[g] (950-1650)	
	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	48 h survival	2450 (LC_{50})[g] (1960-2990)	Bywater *et al.* (1991)
					72 h survival	1665 (LC_{50})[g] (1280-2170)	
					92 h survival	1665 (LC_{50})[g] (1280-2170)	
	Buffalo Billabong	6.4 ± 0.1	3.2 (3.0-3.4)	3 (2.8-3.2)	336 h (14 d) survival	1000 (NOEC)[e] 2040 (LOEC)[f]	Holdway (1992)
					336 h (+ 360 h post exposure)	502 (NOEC)[e] 1000 (LOEC)[f]	

Table 1.3 Cont'd

Species	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Test Endpoint	Water Concentration (µg U L^{-1})	Reference
Purple-spotted gudgeon (*Mogurnda mogurnda*)	Buffalo Billabong	6.3 ± 0.2	4.1 (4.0-4.2)	1.8 (1.7-1.9)	168 h (7 d) survival	1810 (LC_{50})[g] (1730-1780)	Holdway (1992)
					168 h (+ 168 h post exposure)	1015 (LC_{50})[g] (900-1190)	
					168 h (7 d) growth	920 (NOEC)[e] 1780 (LOEC)[f]	
					168 h (+ 168 h post exposure)	< 455 (NOEC)[e] 455 (LOEC)[f]	
	Buffalo Billabong	6.6 ± 0.2	5.1	3.2	96 h survival	1790 (LC50)[g] (1385-2100)	Holdway (1992)
					96 h growth	640 (NOEC)[e] 1240 (LOEC)[f]	
	Buffalo Billabong	6.3 ± 0.2	5.1	3.2	96 h survival	3750 (LC_{50})[g] (2580-4925)	Holdway (1992)
					168 h (7 d) survival	3070 (LC_{50})[g] (2580-3590)	
					168 (+168 h post exposure)	1640 (LC_{50})[g] (1120-2565)	
					168 h growth	2580 (NOEC)[e] 4930 (LOEC)[f]	
					168 h (+ 168 h post exposure)	1240 (NOEC)[e] 2580 (LOEC)[f]	

Table 1.3 Cont'd

Species	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Test Endpoint	Water Concentration (µg U L^{-1})	Reference
Purple-spotted gudgeon (*Mogurnda mogurnda*)	Buffalo Billabong	6.6 ± 0.2	5.1	3.2	96 h survival	3750 (LC_{50})[g] (2580-4925)	Holdway (1992)
					168 h (7 d) survival	3750 (LC_{50})[g] (2580-4925)	
					168 (+168 h post exposure)	3078 (LC_{50})[g] (2580-3590)	
	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	96 h survival	1270 (BEC_{10})[b] 1300 (MDEC)[b] 1570 (LC_{50})[g] (1510-1630)	Markich & Camilleri (1997)

[a] All numerical values represent mean values, or their range, with 95% confidence intervals (C.I) in parentheses (where reported). Means shown with ± values were regulated within the reported limits. NR: not reported. Uranium (U) concentration is expressed as uranyl (ie. UO_2); this was derived by multiplying the U concentration by 1.14.

[b] BEC_{10}, 10% bounded-effect concentration (Hoekstra and van Ewijk, 1993), an analogous statistical measure of the no-observed effect concentration (NOEC).

[c] MDEC, minimal detectable concentration (Ahsanullah and Williams, 1991), an analogous statistical measure of the lowest-observed effect concentration (LOEC).

[d] EC_{50}, median effect concentration.

[e] NOEC, no-observed effect concentration.

[f] LOEC, lowest-observed effect concentration.

[g] LC_{50}, concentration at which there is 50% survival.

The toxicity of U to *M. mogurnda* appears to be very similar in natural creek water and synthetic water, when a similar life stage is used (ie. sac-fry and/or larvae) (Bywater *et al.*, 1991; Holdway, 1992; Markich and Camilleri, 1997). Such a result is interesting given the synthetic water lacks a DOC component, and DOC is considered an influential factor on metal toxicity. The sensitivity of *M. mogurnda* sac-fry to U in natural creek water has also been investigated over longer exposure (ie. 14 d) periods (Holdway, 1992). It was found that *M. mogurnda* sensitivity to U did not necessarily increase with increasing exposure time up to 14 d. Furthermore, *M. mogurnda* mortality was significantly ($P \leq 0.05$) delayed when placed in 'clean' water (ie. at natural U concentration) for 15 d after being exposed to U for 14 d.

1.3.4 Mechanisms of U toxicity in water

To prevent metal toxicosis, aquatic organisms integrate their excretion and storage processes to manage metal uptake. Some organisms are able to regulate the levels of a particular metal in their bodies independently of environmental concentrations, while others accumulate the metal in their bodies, detoxifying when necessary (Hyne *et al.*, 1993).

Freshwater hydra, such as *H. viridissima*, are particularly sensitive to metals as they lack metal-binding proteins which sequester and detoxify metals (Hyne *et al.*, 1993). The symbiotic algae hosted by *H. viridissima* help regulate exposure to elevated levels of a metal by accumulating the metal, and if necessary shedding it from the host tissue (Hyne *et al.*, 1993). Uranium has been found to accumulate in nematocysts of hydra and inhibit the replacement of discharged nematocysts, resulting in feeding dysfunction and reduced population growth (Hyne *et al.*, 1993). The walls of the nematocyst capsules are collagenous in nature (Blanquet and Lenhoff, 1966) and like many other collagens may have an affinity for U (Anselme *et al.*, 1990).

In higher animals, the mechanism of U toxicity may be attributed to changes in cellular membrane permeability due to the binding of uranyl ions to phosphate ligands and to the inhibition of cellular carbohydrate metabolism (Ellender *et al.*, 1992). The principle effect is the inactivation of phosphate-containing molecules and biological ligands such as ATPase (Ellender *et al.*, 1992). Refer to Section 1.2.4 for other generic mechanisms already outlined for Cu (eg. hardness, pH etc.).

1.3.5 Effect of physico-chemical parameters on U toxicity

Increasing water hardness and alkalinity are typically considered to reduce the toxicity of U to freshwater organisms (Tarzwell and Henderson, 1960; Poston *et al.*, 1984; Parkhurst *et al.*, 1984; Barata *et al.*, 1998). However, such studies failed to define the effects of true water hardness (ie. Ca and/or Mg concentration) independently of alkalinity and pH. For

example, Parkhurst *et al.* (1984) described the 96 h LC_{50} of U in hard water (208 mg L^{-1} hardness as $CaCO_3$; 53 mg L^{-1} alkalinity as $CaCO_3$; pH 7.5) to be approximately four-fold greater than in soft water (35 mg L^{-1} hardness as $CaCO_3$; 11 mg L^{-1} alkalinity as $CaCO_3$; pH 6.7) for juvenile Brook trout (*Salvelinus fontinalis*). Parkhurst *et al.* (1984) described the relationship between hardness and toxicity as a function of carbonate alkalinity, which was supported by Poston *et al.* (1984) using *D. magna*.

Under constant water hardness and pH conditions, alkalinity has been found to attenuate the effects of U toxicity to a freshwater bivalve (*Velesunio angasi*) (Markich *et al.*, 1996). In support of this relationship, geochemical speciation modelling found U toxicity to be inversely proportional to the percentage of UO_2CO_3 in solution, implying UO_2CO_3 is not toxic. Several studies agree that uranyl complexes are less toxic than UO_2^{2+} (Nakajima *et al.*, 1979; Poston *et al.*, 1984; Greene *et al.*, 1986). Poston *et al.* (1984) proposed that U toxicity is ameliorated due to an increase in the formation of uranyl carbonate complexes reducing the free hydrated uranyl ion (UO_2^{2+}) concentration. Markich and Jeffree (1994) suggested U toxicity is reduced by Ca^{2+} and/or Mg^{2+} ions competing with UO_2^{2+} for binding and transport sites at the cell membrane, without directly altering U speciation in water. The effect of true water hardness on the toxicity and bioavailability of U to freshwater biota has yet to be described.

Few studies have examined the effects of pH on U toxicity. Those that have have used different test organisms making it difficult to directly compare studies. An autonomous increase in pH over a range of 2.0 to 7.0 has been reported to reduce U toxicity (Nakajima *et al.*, 1979; Greene *et al.*, 1986; Markich *et al.*, 1996). For example, Markich *et al.* (1996) found a decrease in pH from 6.0 to 5.0 to have a five-fold increase in U toxicity to a freshwater bivalve, *Velesunio angasi* in synthetic Magela creek water. The enhancing effects of pH on the toxicity of U were supported by large changes in U speciation, as predicted by geochemical speciation modelling (Markich *et al.*, 1996). Nakajima *et al.* (1979) and Greene *et al.* (1986) suggest that low pH inhibited the binding of U to *Chlorella sp.* by protonation of weak, basic binding sites on the algal surface. In contrast to the response of *V. angasi* to U (Markich *et al.*, 1998), Franklin *et al.* (1998) observed that a decrease in pH from 6.5 to 5.7 had a two-fold reduction in U toxicity to a freshwater alga, *Chlorella sp.* in synthetic Magela creek water. The notion that H^+ in solution is able to elicit a protective effect is gathering support (Crist *et al.*, 1988; Schenck *et al.*, 1988; Parent and Campbell, 1994). It has been proposed that the H^+ concentration either directly affects metal uptake or indirectly affects the chemical speciation of the dissolved metal (Franklin *et al.*, 1998). Uncoupling these two factors is necessary to correctly understand U toxicity and bioavailability to freshwater biota.

Controversy surrounds the effect hydrophilic organic ligands exert on the toxicity and bioavailability of U in aquatic systems. Uranium toxicity was found to decrease in the presence of organic ligands (model fulvic acid), by complexing cationic uranyl species (eg. UO_2^{2+} and UO_2OH^+) (Yong and Macaskie, 1995; Markich *et al.*, 1996). In contrast, the complexation of uranyl with oxalate ($[UO_2(Ox)_2]^{2-}$) was found to enhance U toxicity to a lichen, *Cladonia rangiferina* (Boileau *et al.*, 1985).

1.4 Aim of study

The aim of this study was to separate the effects of true water hardness (Ca and Mg) and alkalinity (carbonate), at a constant pH, on the toxicity of Cu and U to *H. viridissima* (Green hydra, population growth) and *M. mogurnda* (Purple-spotted gudgeon, sac-fry survival). This study also attempted to investigate the effects of pH (proton concentration), at constant hardness and alkalinity, on the toxicity of Cu and U to *H. viridissima* and *M. mogurnda* (Refer to Appendix E for details). Gaining a fundamental understanding of how these parameters affect metal toxicity and bioavailability is an essential aspect of site-specific environmental risk assessment and water quality guideline derivation.

2 General materials and methods

The effect of water hardness and alkalinity on the toxicity of Cu and U to *H.viridissima* population growth and *M.mogurnda* sac-fry survival were assessed using existing protocols from the Environmental Research Institute of the Supervising Scientist (Hyne *et al.*, 1996; Markich and Camilleri, 1997). The protocols are detailed in Appendix B, while the general test procedures are outlined below. Specific modifications made to the standard protocols to enable water parameter manipulations are described where necessary.

2.1 Toxicity test media preparation

2.1.1 Preparation of equipment and solutions

All equipment that comes in contact with chemical solutions or test organisms was pre-cleaned using detergent (2% Neutracon), followed by nitric acid (5% AnalaR) and then deionised water (Milli-Q, <1 $\mu S\ cm^{-1}$), to avoid contamination. Plasticware was used to avoid the adsorption of metals. All bottles and vials used for chemical analysis were prepared in the same manner. All reagents used were analytical grade.

2.1.2 Preparation of diluent water

Toxicity tests used a 'synthetic' water that simulates the inorganic composition of a tropical Australian sandy-braided stream during the Wet Season. More specifically, the synthetic water quality characteristics are based on Magela Creek water (Alligator Rivers Region, Northern Territory) (Figure 2.1). This water is very soft (2-4 mg L^{-1} as $CaCO_3$), slightly acidic (mean pH 6.0), with a low buffering and complexation capacity (Markich, 1998). The inorganic component is used to provide a maximum risk scenario to assess the potential impact of metals to aquatic organisms. Organic chelating agents (ie. DOC) are excluded from the synthetic media, as metals complex with DOC, and their toxicity is subsequently ameliorated (Meador, 1991). Using a standard water chemistry also provides a baseline from which a large range of different water quality parameters could be calibrated and assessed.

The synthetic water solution was prepared in 20 L volumes and the pH adjusted to 6.0 ± 0.15 using 0.02 M HNO_3, as close as practical to test commencement. The water was stored and aerated in a pre-cleaned polyethylene 25 L container, 24 h prior to preparation of test solutions.

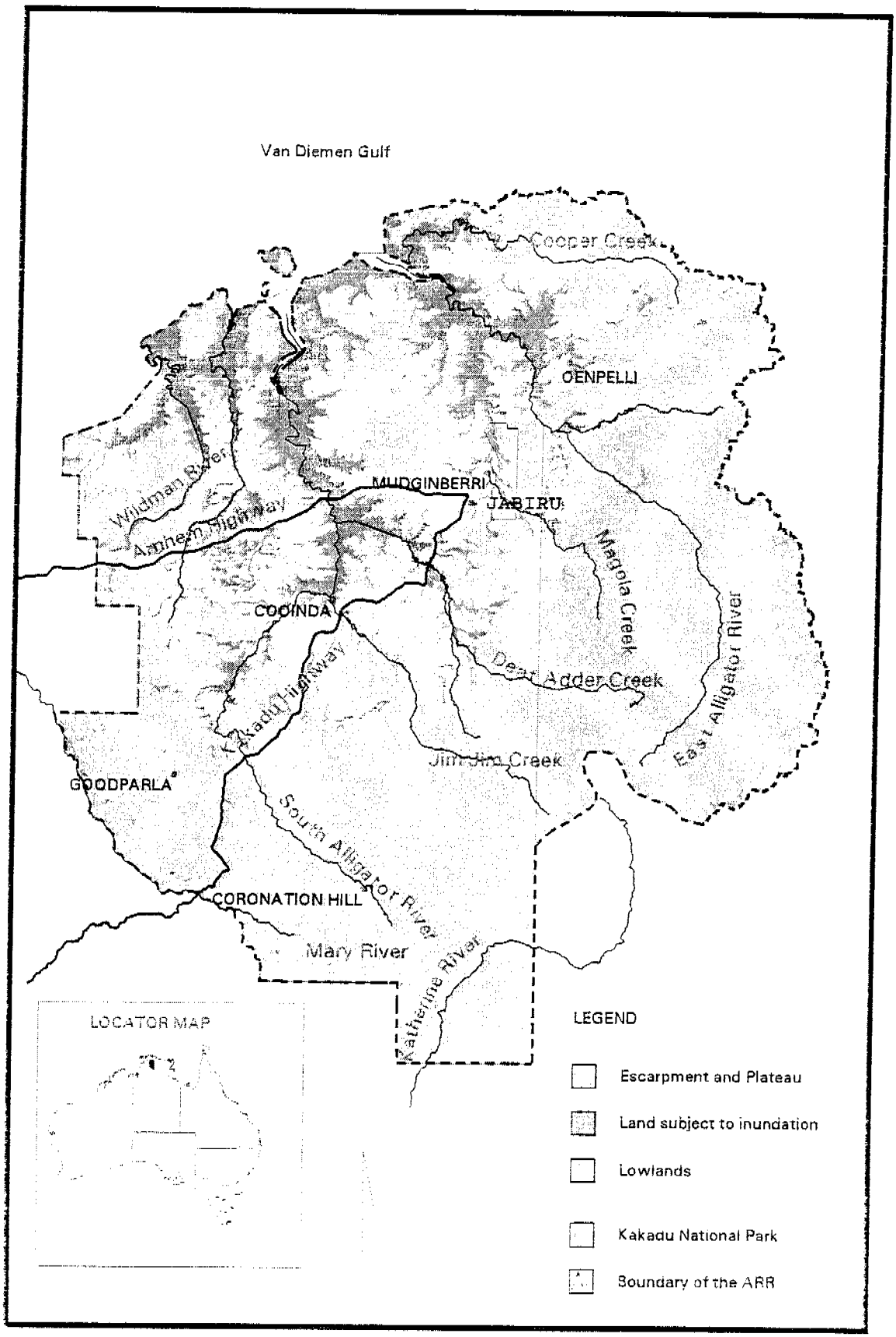

Figure 2.1: Map of the Alligator Rivers Region, Northern Territory, including the Magela Creek catchment.

2.1.3 Preparation of test solutions

A 2000 mg L^{-1} Cu stock solution and a 400 mg L^{-1} U stock solution were prepared using analytical grade $CuSO_4.5H_2O$ and $UO_2SO_4.3H_2O$ in high purity deionised water (Milli-Q). The stock solutions were prepared in pre-cleaned 1L plastic bottles and refrigerated at 4°C for the test duration. Prior to use, stock solutions were allowed to equilibrate to room temperature. Test solutions were prepared by serially diluting respective stock solutions with pH-adjusted synthetic water. Initial range-finding test concentrations were determined from the results obtained by Markich and Camilleri (1997). A bulk quantity of each test solution was prepared in pre-cleaned 2 L polyethylene screw-topped bottles, immediately prior to test commencement. Over the test period, test solutions were maintained at 4°C until required for daily renewal. They were then equilibrated to 27 ± 1°C inside a constant temperature incubator for several hours. Prior to the daily renewal of test solutions, aliquots of each test concentration were dispensed and the pH adjusted to pH 6.0 ± 0.3 using 0.02 M HNO_3 and 0.0125 M NaOH.

2.2 Green hydra (*Hydra viridissima*) population growth test

2.2.1 Bioassay selection

Freshwater cnidaria have several inherent advantages for use in bioassays:

1. Hydra are diploblastic (ie. has only two epithelial layers), so all cells are in contact with the surrounding medium (Beach and Pascoe, 1998);
2. Hydra reproduce asexually, so all the animals in the stock culture are genetically very similar (Beach and Pascoe, 1998);
3. Low genetic variation among test individuals minimizes experimental variation due to differences in morphology, and maximizes experimental reproducibility;
4. Hydra are able to withstand extensive manipulation, and are easily reared and maintained in the laboratory (Blaise and Kusui, 1997);
5. Hydra assays are simple, cost effective, and easy to conduct (Blaise and Kusui, 1997).

2.2.2 Species description

Hydra viridissima (Cnidaria, Hydrozoa) is referred to as 'green hydra' due to the presence of a symbiotic green alga in the gastrodermal cells of the animal. It is the alga that gives the organism its green colouration. The genus distribution is considered ubiquitous throughout Australian freshwater systems (Lesh-Laurie, 1982). The presence of *H. viridissima* in tropical freshwaters of Northern Australia was first reported by Bale (1919). *Hydra viridissima* exhibit a solitary polyp form and are capable of reproducing sexually and asexually by budding. Budding is a characteristic of hydra in optimal environmental

conditions. A bud develops on the stalk as a simple evagination of the body wall (Barnes, 1980). The distal end of the bud forms a mouth encircled by tentacles; the whole bud then detaches from the parent to form an individual hydra (Barnes, 1980). *Hydra viridissima* is considered the most ecotoxologically relevant species of freshwater hydra to use in a bioassay. The sensitivity of *H. viridissima* to U has been found to be 3-4 times greater than the pink hydra, *Hydra vulgaris* (Appendix A).

2.2.3 Stock culture maintenance

Hydra viridissima were originally collected from Magela Creek (Figure 2.1). A primary stock was cultured in the laboratory in aerated 2 L glass bowls containing synthetic water. A secondary stock of hydra was maintained in a tap-water filled 'community' aquaria in a separate location, as a precaution against contamination or accidents. Both stock cultures were fed and cleaned regularly, as detailed in Appendix C.

2.2.4 Selection of hydra for test commencement

Hydra free of overt disease or gross morphological deformity were considered suitable test organisms. Such hydra were obtained from laboratory cultures. Each test was initiated using hydroids bearing one tentacled bud. A hydroid is defined as a single polyp of the coelenterate.

2.2.5 Test procedure

Asexually reproducing (budding) hydra were exposed to a range of Cu or U concentrations for a period of 96 h. To commence the test, 30 mL of each test concentration was aliquoted into 40 mL Petri dishes and ten hydra were randomly placed in each Petri dish. Three replicates were used for each test concentration. The test dishes were kept in a constant temperature incubator at 27 ± 1°C, with a photoperiod of 12 h light and 12 h dark, for the duration of the test period. At each 24 h interval the number of intact hydroids was recorded, where one hydroid equalled a single animal plus any attached buds. The physical features of tentacle clubbing and contraction were recorded and used as qualitative test endpoints, indicating whether the hydra was in sub-optimal conditions. Each hydroid was individually fed with 3-4 live brine shrimp nauplii (*Artemia franciscana*) per 24 h period. The hydra were allowed to feed for approximately 2 h before the test solutions were renewed. Solution renewal involved transferring the hydra and their progeny to fresh test media at each 24 h interval. The pH, conductivity and dissolved oxygen of test waters were measured at the commencement and conclusion of daily water renewal. After 96 h the test was terminated and the quantitative population growth response was statistically analyzed. The test was considered valid if control population growth was greater than 20 individuals after 96 h. Appendix B describes the protocol in more detail.

2.3 Purple-spotted gudgeon (*Mogunda mogurnda*) sac-fry survival test

2.3.1 Bioassay selection

The value of assessments using fish, identified by Harris (1995), are summarised here:

1. Fish communities represent various trophic classes and use foods from aquatic and terrestrial sources, providing an integrative view of the watershed;
2. Both acute toxicity (fish mortality) and stress effects (depressed growth or reproductive success) can be evaluated;
3. Fish are primarily affected by macro-environmental influences (eg. water quality and energy source), unlike algae and macroinvertebrates that are affected by both micro- and macro-environmental influences;
4. Being relatively long-lived, fish provide temporal integration in assessments.

The present study used *M. mogurnda* sac-fry, as eggs or early life stages are usually more sensitive to toxicants than adults (Kong *et al.*, 1995).

2.3.2 Species description and husbandry

Mogurnda mogurnda (Teleostomi, Eleotrididae) is commonly known as purple-spotted or northern trout gudgeon (Merrick and Schmida, 1984). The freshwater species has a wide distribution throughout Northern Australia, extending south to Lake Eyre and northeast coast regions (Merrick and Schmida, 1984). *Mogurnda mogurnda* are carnivorous and the sexes are dimorphic (Merrick and Schmida, 1984). Fertilisation is external, with the female laying a batch of 300-1000 eggs.

Reproductive juvenile gudgeons were collected from local waterways within the Magela Creek system of the Alligator Rivers Region, Northern Territory. Fish were captured by baited fish traps and brought back to the aquaculture facility at the Environmental Research Institute of the Supervising Scientist. The fish were sexed, divided into compatible breeding groups of one male and two females, and placed in 420 L aquaria. The temperature of the aquaria was monitored and the fish were fed on a daily basis. Appendix D recommends husbandry methods that provide optimal breeding conditions to ensure the continuous production of sac-fry to conduct toxicity tests.

2.3.3 Isolation and selection of sac-fry for test commencement

It is the sac-fry lifestage of *M. mogurnda* that is used in the toxicity test protocol. When a batch of eggs is produced, they are left in the parent tank for 24-48 h allowing the male parent fish to guard them. After this time, the developing embryos are carefully removed by placing the object on which they are laid into a 2 L glass beaker containing ½ parent tank

water and ½ diluent water. The beaker is then placed on a warming tray set at 27 ± 1°C in the temperature controlled laboratory to continue development. Gentle aeration is used to simulate the male parent 'fanning' water over the eggs to reduce the incidence of fungal spores settling. The eggs hatch after 3-4 d. Once all the eggs have hatched (or at least sufficient numbers to enable a test to be started), they are carefully isolated into a Petri dish. Neither the embryos nor sac-fry are treated for fungus. Examination is made under a microscope to determine which sac-fry are free from overt disease or gross morphological deformity and are suitable test organisms.

2.3.4 Test procedure

Recently hatched sac-fry (<10 h old) were exposed to a range of Cu or U concentrations for a period of 96 h. To commence the test, 30 mL of each test concentration was aliquoted into 40 mL Petri dishes and ten sac-fry were randomly placed in each Petri dish. The test dishes were kept in a constant temperature incubator at 27 ± 1°C, with a photoperiod of 12 h light: 12 h dark, for the duration of the test period. At each 24 h interval the number of surviving sac-fry was recorded (ie. sac-fry with a heartbeat). Changes in morphology and the presence of fungus were recorded and used as qualitative test endpoints, indicating whether the sac-fry were in sub-optimal conditions. The sac-fry did not require feeding prior to, or during, the 96 h test period, as the animals obtain sufficient nutrition from the attached yolk sac. Every 24 h the test solutions were renewed and the surviving sac-fry transferred to the fresh solutions. The pH, conductivity and dissolved oxygen of test waters were measured at the commencement and conclusion of daily water renewal. After 96 h the test was terminated and the quantitative survival response statistically analysed. The test was considered valid if control mortality did not exceed 20% after 96 h. Appendix B describes the protocol in more detail.

2.4 Chemical analysis

2.4.1 Physico-chemical analysis of test solutions

The pH and conductivity of the test solutions were measured using an Alpha® pH/conductivity meter. A combination pH electrode (Sensorex®) was calibrated daily with standard buffer solutions (BDH®). A platinum/glass conductivity cell (EDT®) was calibrated daily with a standard potassium chloride solution. The dissolved oxygen concentration was measured using a polarographic electrode coupled to an Activon® (Model 401) oxygen meter. The pH, conductivity and dissolved oxygen of fresh (t_0) and 24 h-old (t_{24}) treatment solutions were measured for the duration of a test. The pH was adjusted to pH 6.0 ± 0.3 when required.

Alkalinity was determined using a potentiometric titration method, as outlined by APHA *et al.* (1998) (Section 2320B.4d). A Metrohm® 682 Titroprocessor was used to perform the titrations. The alkalinity of treatment solutions was calculated at the start (t_0) and end (t_{96}) of a test. The measured alkalinity was typically within 10% of the nominal alkalinity.

Treatment solutions were sub-sampled and analysed for a range of elements using a combination of analytical techniques. The concentrations of Ca, Mg, Na, and K were measured on unacidified (4mL) samples by high performance liquid chromatography (HPLC). Concentrations of Cu and U were measured by inductively coupled plasma mass spectrometry (ICP-MS) on acidified (pH<2), indium spiked 50 mL samples. The measured concentrations of Cu and U were within 15% of the nominal concentrations, but typically less than 5%. Measured concentrations of Cu and U were used to calculate dose-response curves and perform statistical analyses.

Uranium is present in the environment in several oxidized states. In oxidized waters it is the hexavalent state (UO_2^{2+}; uranyl ion) which predominates, and therefore, has been used to represent U in this study (Markich and Camilleri, 1997).

2.4.2 Geochemical speciation modelling of Cu and U

The speciation of a metal in solution determines its bioavailability, and consequently, its toxicity to aquatic biota. The thermodynamic geochemical speciation code HARPHRQ (Brown *et al.*, 1991) was used to predict the speciation of Cu and U in the test solutions. The input parameters for HARPHRQ were based on physico-chemical data measured from the treatment solutions. Stability constants used in HARPHRQ are given in Markich *et al.* (1996). Information derived from HARPHRQ was used to assist in the interpretation of the toxicity test results.

2.5 Statistical analysis

Toxicity tests involving *H. viridissima* investigated a single hardness or alkalinity level per test, with 3-4 replicates per metal concentration. *Mogurnda mogurnda* toxicity tests investigated all hardness levels within a single test, with 2-3 replicates per metal concentration (Refer Chapter 3.2.3). Population growth and survival were measured as a percentage of control, where the control response equaled 100%. Data derived for each hardness or alkalinity level was pooled and a mean and 95% confidence interval (C.I.) calculated. These results were plotted against measured metal concentrations to derive a sigmoidal dose-response curve. The curve was fitted using the software package, Origin© (Version 4.1). Using the model, the EC_{50} or LC_{50} and 95% C.I. was calculated. The data for each test were also entered into Minitab® and an ANOVA (ie. analysis of variance) performed. Tukey's comparison test was also performed to determine which treatments

were significantly different from one another (α = 0.05). This information enabled estimation of the NOEC and LOEC. The BEC_{10}, an alternative to the NOEC, was estimated using the approach described by Hoekstra and van Ewijk (1993b). The BEC_{10} being the highest concentration for which can be claimed with 95% confidence that its biological effect does not exceed 10% of the observed effect (Hoekstra and van Ewijk, 1993b). The process of deriving the BEC_{10} may not utilise all the data as point estimation does. Instead, it involves calculating the BEC_{25}, which is the concentration whose upper/lower 95% confidence limit does not exceed 25% of the observed effect, and subsequent linear extrapolation to the 10% effect level (ie. the BEC_{10}). The MDEC, an alternative to the LOEC, was estimated using the approach described by Ahsanullah and Williams (1991). The MDEC was calculated from a regression model and is defined as the metal concentration at which the response became significantly lower than in the 'control' treatments.

3 Effect of true water hardness on the toxicity of Cu and U

3.1 Rationale

The hardness of fresh surface water is known to influence the bioavailability of metals to aquatic organisms. Quantitative relationships (algorithms) have been established to describe the reduction in the bioavailability of Cd, Cr(III), Cu, Ni, Pb and Zn as a function of increasing water hardness. These algorithms have been incorporated into the water quality guidelines of several countries for the protection of aquatic ecosystems (CCREM, 1991; US EPA, 1995; ANZECC and ARMCANZ, 1999). Although several studies have found that water hardness typically reduces the toxicity of U to freshwater biota (eg. Parkhurst *et al.* 1984; Barata *et al.* 1998) (all temperate Northern Hemisphere species), insufficient and/or inconsistent data have precluded an algorithm being established (as detailed in Section 1.2.5 and 1.3.5).

In brief, previous studies that have investigated the influence of water hardness on the toxicity of metals to freshwater biota have confounded the effects of true water hardness (ie. Ca and/or Mg concentration) with alkalinity (ie. carbonate concentration) and pH (ie. proton concentration), since an increase in water hardness is frequently associated with an increase in alkalinity (where Ca and/or Mg are added as carbonate) and pH (Stumm and Morgan, 1981). It is important to separate the effects of hardness and alkalinity, since each variable has a different mechanism of toxicity. Calcium and/or Mg competitively inhibit the uptake, and hence, toxicity of trace metals at the cell membrane surface (Markich and Jeffree 1994), whereas complexation of trace metals with carbonate in the aquatic medium, reduces the concentration(s) of toxic metal species (ie. a change in metal speciation) (Hunt, 1987).

Thus, one objective of this study was to isolate and assess the effects of true water hardness, at constant alkalinity and pH, on the toxicity of Cu and U to *H. viridissima* (Green hydra, population growth) and *M. mogurnda* (Purple-spotted gudgeon, sac-fry survival).

3.2 Methodology

Toxicity testing materials and procedures are detailed in Chapter 2. Only specific modifications made to these standard protocols are mentioned here.

3.2.1 Selection of water hardness levels

Regional water quality information was gathered from Northern Territory Water Resources and the Environmental Research Institute of the Supervising Scientist to determine a relevant range of hardness for tropical Australian freshwater systems (Figure 3.1; Table 3.1).

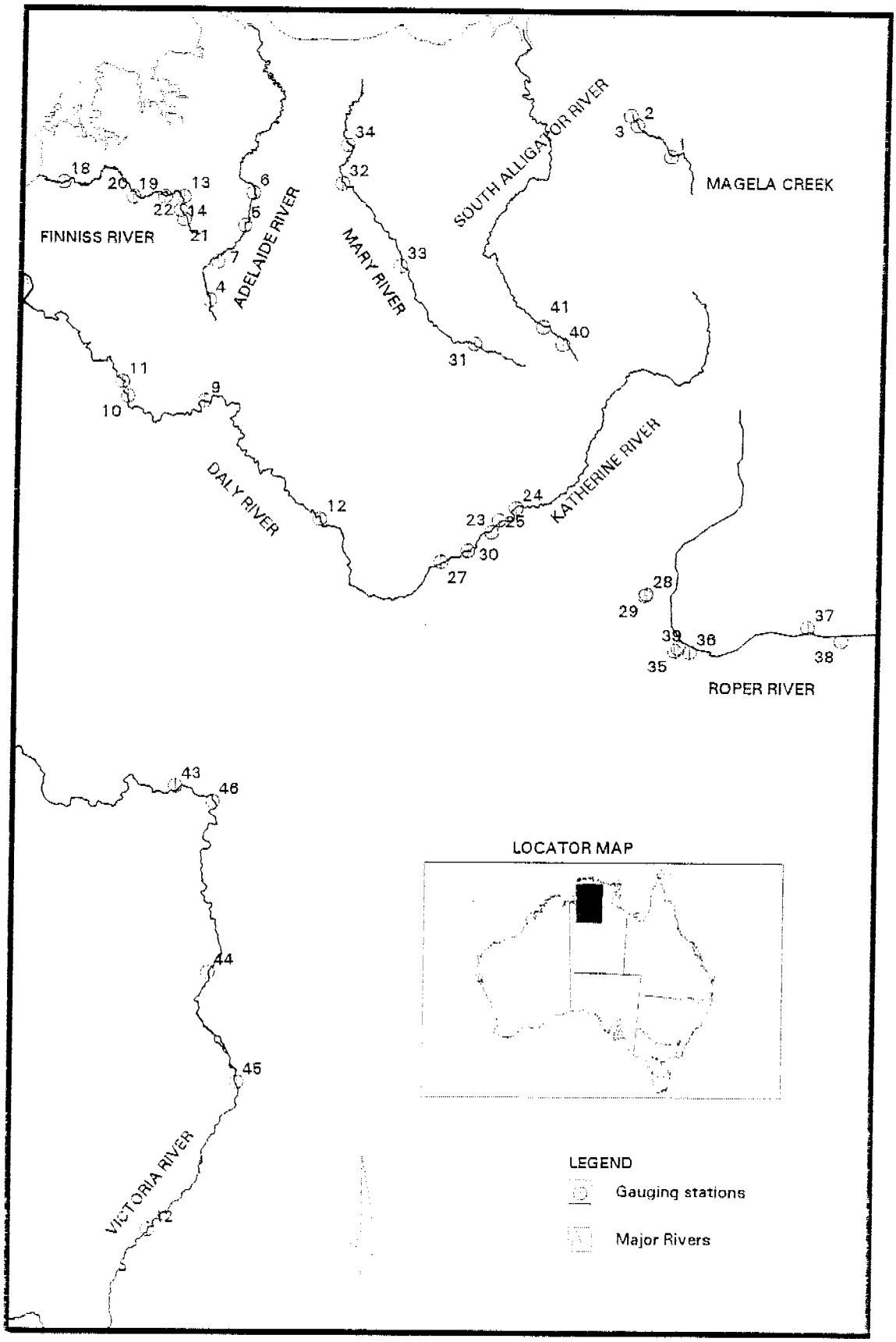

Figure 3.1: Location of gauging stations along several Northern Territory water systems. Gauging station numbers correspond to Table 3.1.

Table 3.1: Mean pH, hardness and alkalinity of Northern Australian river systems between 1962-1997. Data supplied by Northern Territory Water Resources and the Environmental Research Institute of the Supervising Scientist. Station number corresponds to Figure 3.1.

Station No.	Station Name	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})
1	Magela Ck at Bowerbird billabong	6.1	5.3	2.3
2	Magela Ck (near *eriss*)	6.02	5.7	3.71
3	Magela Ck at Mudjenberri Billabong	6.22	5.4	4.31
4	Adelaide River 8 km downstream of Daly Road	6.9		45.17
5	Adelaide River at Tortilla Flats	7.3	92	9303
6	Adelaide River upstream Marrakai Crossing	6.83	83	93.32
7	Adelaide River at Railway Bridge	7.1	74.67	54.61
9	Daly River at Beeboom Crossing 2km downstream	8.25		259.44
10	Daly River at Mount Nancar	7.75	18	154.98
11	Daly River at Police Station	8.2		199.73
12	Daly River at upstream Dorisville Crossing	7.83	218	206.63
13	East Finniss River at Rum Jungle	5.96	68.5	8.65
14	Finniss River at Batchelor Damsite	7.13	109	140.52
18	Finniss River at Point 1	6.2		89
19	Finniss River at Point 10	6.93	58.14	50.38
20	Finniss River at Point F (Bad Crossing)	5.75	5.5	14.12
21	Finniss River at Point L	7.23	58	90
22	Finniss River at Taw 2	6.4		115.67
23	Katherine River at Donkey Camp Outflow	7.05	9.5	12.29
24	Katherine River at Gorge Caravan Park	6.1		11.05
25	Katherine River at inflow to Donkey Camp	6.3	7	9
27	Katherine River at PT"Am"	7.8	370	302
28	Katherine River Site 43	7.9	284	279
29	Katherine River Site 44	7.9	291	281
30	Katherine River downstream Sewage Ponds Outflow	6.85		170.73
31	Mary River at El Sherana Rd Crossing	6.6		19.63
32	Mary River at Mount Bundey	6.45		26.26
33	Mary River at Pt.9a	6.65		22.33

Table 3.1 Cont'd

Station No.	Station Name	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})
34	Rockhole Weedsite at Corroboree - Mary River	5.83	15	12.18
35	Roper River - Mataranka Homestead Crossing	6.5	36	328.25
36	Roper River at downstream Mataranka Homestead	7.8	477	271.14
37	Roper River at downstream Moraok Homestead	8.2		284.8
38	Roper River at Mole Hill	7.25	252	199
39	Roper River at Thermal Springs Mataranka	7.1	481	447.89
40	South Alligator River at Coronation Hill	7.05	50	51.75
41	South Alligator River at El Sharana ("c")	7.7	53	35.91
42	Upper Victoria R. at Wave Hill Police Station	7.70	210	173.14
43	Victoria River at Coolibah Homestead	8.15	219.25	206.36
44	Victoria River at Dashwood Crossing	7.95	258.5	189.13
45	Victoria River at Pigeon Hole Homestead	7.77	1.8	146
46	Victoria River at Victoria Highway	8.10	1.85	211.13

Three levels of hardness were selected - 6.6, 165 and 330 mg L^{-1} as $CaCO_3$. The baseline hardness of 6.6mg L^{-1} $CaCO_3$ represents the hardness of Magela Creek water (Refer Figure 3.1; Table 3.1). The rationale for using Magela Creek water as a baseline reference in this study is outlined in Appendix B.1.4. The other hardness levels were based on a linear scale to complete a representative range of fresh surface waters in tropical Australia (Refer Figure 3.1; Table 3.1).

3.2.2 Isolating hardness effects

True water hardness was achieved by adding $Ca(NO_3)_2$ and $Mg(NO_3)_2$ to the synthetic diluent water, while other physico-chemical parameters were held constant (ie. pH 6.0 ± 0.3 and conductivity within 10% error, over 24 h). Calcium and Mg were added as nitrate since this anion forms a very weak complex with Cu and U, and hence, minimises speciation changes.

Two preliminary tests were conducted to ensure test organism reproduction and/or survival were not affected by the addition of $Ca(NO_3)_2$ and $Mg(NO_3)_2$ to increase water hardness to 165 mg L^{-1} and 330 mg L^{-1} $CaCO_3$. The population growth of *H. viridissima* and *M. mogurnda* sac-fry survival in treatments with 165 and 330 mg L^{-1} hardness, were compared to growth in 6.6 mg L^{-1} hardness, using the protocols described in Appendix B. In synthetic water at 6.6 mg L^{-1} hardness, *H. viridissima* are expected to at least double their population growth, while *M. mogurnda* sac-fry survival is expected to be ≥ 80%, over 96 h (Appendix B.1.13 and B.2.13). It was clear that *H. viridissima* population growth and *M. mogurnda* sac-fry survival were not affected by the addition $Ca(NO_3)_2$ and $Mg(NO_3)_2$ (Table 3.2).

Table 3.2: Population growth of *H. viridissima* and survival of *M. mogurnda* sac-fry following 96 h exposure to three hardness levels (ie. 6.6, 165 and 330 mg L^{-1} $CaCO_3$). The number of organisms at 0 h equaled ten per treatment.

Hardness (mg L^{-1})	*H. viridissima* population growth				*M. mogurnda* sac-fry survival			
	Rep 1	Rep 2	Rep 3	Mean	Rep 1	Rep 2	Rep 3	Mean
6.6	37	37	30	35	10	10	10	10
165	32	39	33	35	10	10	10	10
330	37	36	34	36	10	10	10	10

3.2.3 Change to *M. mogurnda* protocol to allow water parameter manipulation

While using the established protocol described in Appendix B.2, to investigate the effects of hardness on Cu and U toxicity to *M. mogurnda*, it became apparent that variability in sac-fry health and genetics (ie. parent stock) produced large variability around toxicity endpoints (eg. LC_{50}). Consequently, the effect of hardness on metal toxicity was obscured. Such variability was not observed for *H. viridissima*. To reduce the influence of biotic variables, sac-fry from a single parent pair were used to examine the effect of all hardness levels in one experiment, instead of using the single batch of sac-fry to investigate one hardness level per experiment. The underlying rationale for this change in method was that batch variability would only occur between experiments, instead of within experiments and hardness levels. The revised method provided a more reliable dose-response curve, and therefore, a more confident estimate of the effect of hardness on Cu and U toxicity to *M. mogurnda*. For this method to be logistically possible, while remaining scientifically sound, the number of replicates per experiment were reduced from three to two, and 3-4 experiments were performed to generate an LC_{50} value.

3.2.4 Assessment of hardness effects on U toxicity to *M. mogurnda* sac-fry

The effect of hardness on the toxicity of U to *M. mogurnda* was assessed over two time periods (January-February, 1998 and February-April, 1999). It was considered necessary to investigate this section of study for a second time, to validate an anomalous result from the initial investigation. Further details are provided in the Discussion (Section 3.4).

3.3 Results

This study was designed to assess the effects of true water hardness (Ca and Mg concentration) on the toxicity of Cu and U to *H. viridissima* and *M. mogurnda*, at constant alkalinity (4 mg L^{-1} as $CaCO_3$) and pH (6.0 ± 0.3). Raw data for each test-series of a given metal-organism exposure are provided in Appendix F, Tables 1-4.

3.3.1 Influence of hardness on Cu toxicity

The concentration-response relationships for *H. viridissima* and *M. mogurnda* exposed to Cu at three hardness levels are shown in Figures 3.2 and 3.3, respectively. Summary data for each concentration-response curve are given in Appendix G, Table 1 and 2. The calculated BEC_{10}, MDEC, NOEC, LOEC, EC_{50} and LC_{50} values for *H. viridissima* and *M. mogurnda* exposed to Cu at three hardness levels (6.6, 165 and 330 mg L^{-1} as $CaCO_3$), are given in Table 3.3.

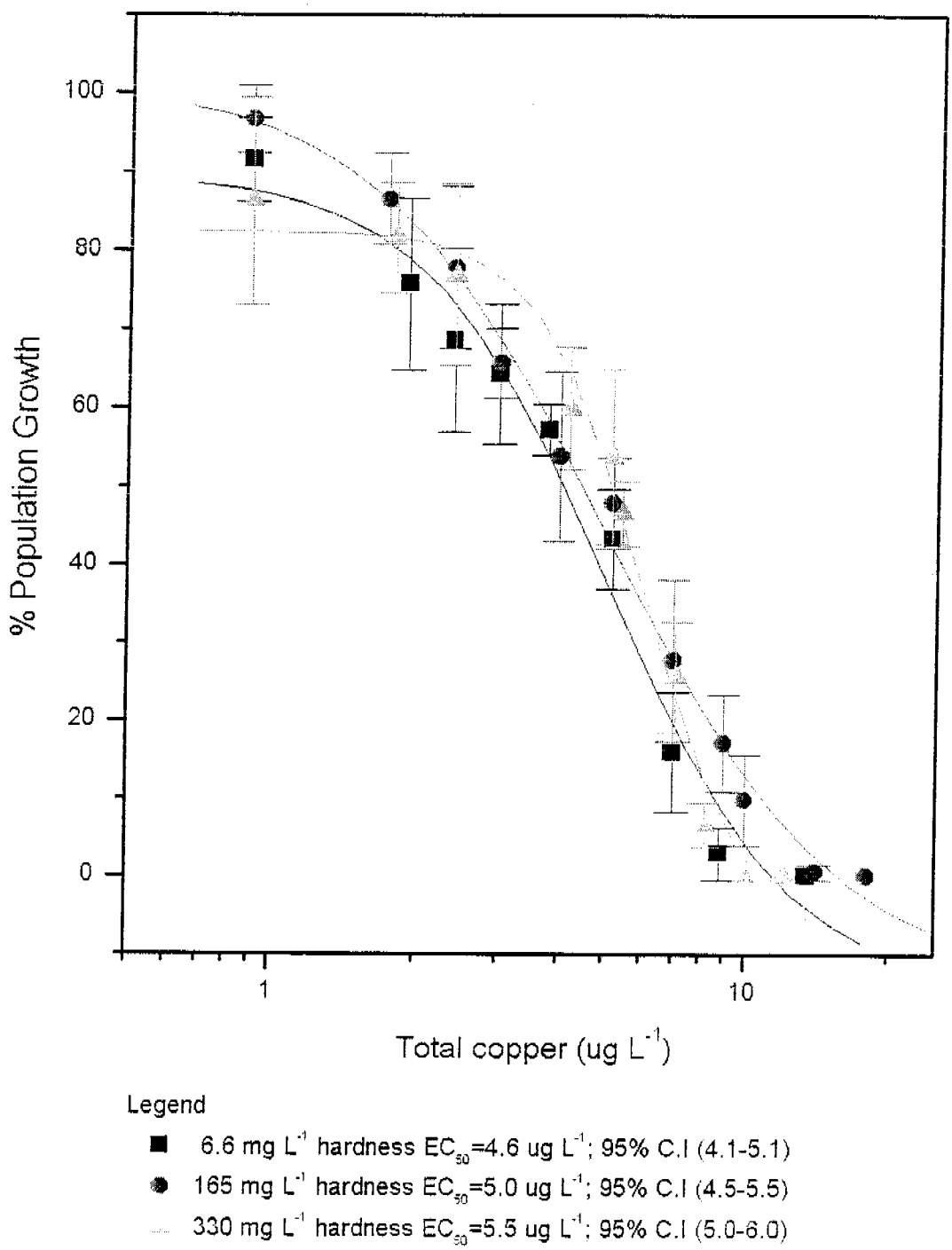

Figure 3.2: Population growth of *H. viridissima* exposed to Cu over 96 h at three hardness levels (6.6, 165 and 330 mg L^{-1}). Data points represent the mean of six or nine replicates ± 95% C.I.

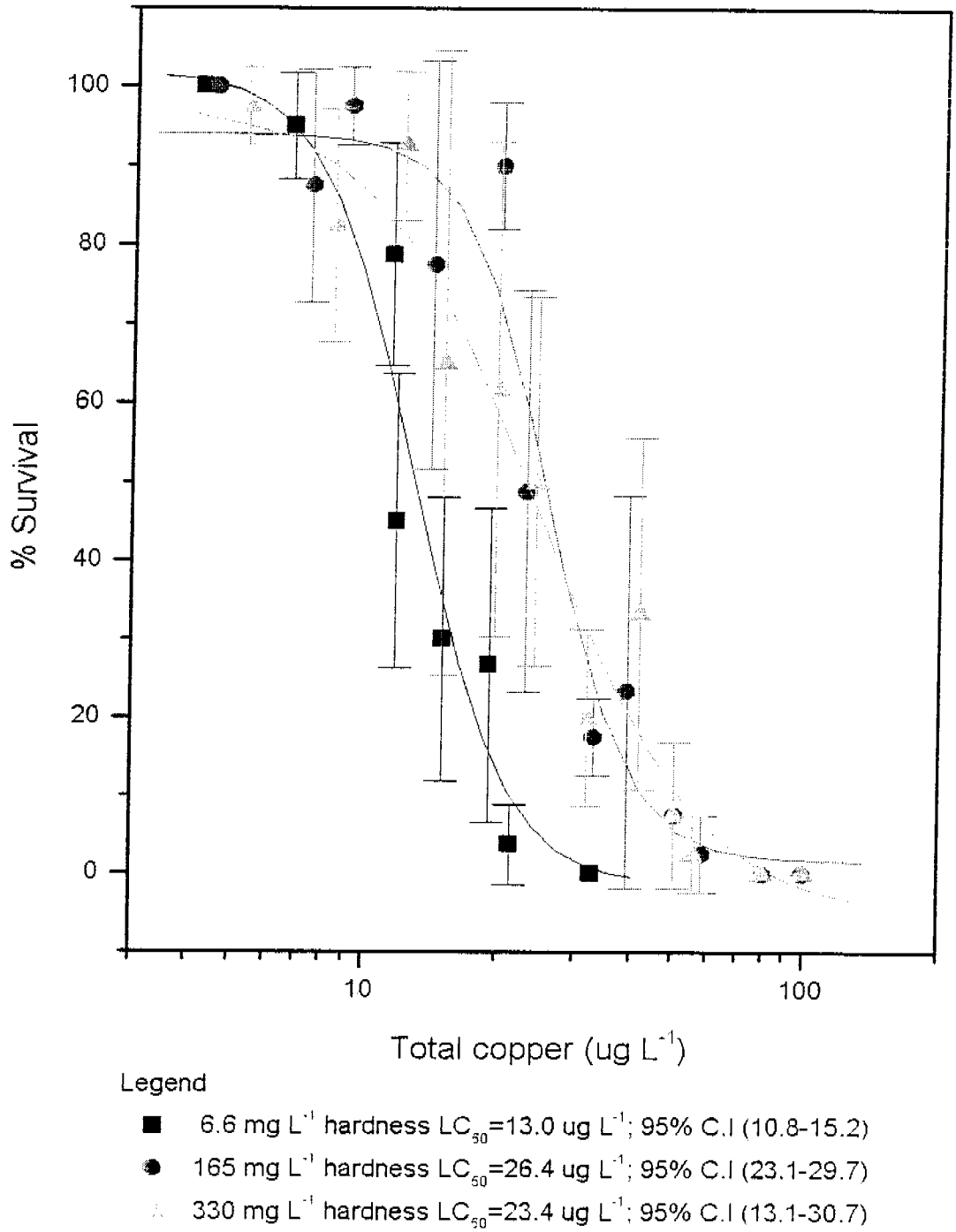

Figure 3.3: Survival of *M. mogurnda* exposed to Cu over 96 h at three hardness levels (6.6, 165 and 330 mg L^{-1}). Data points represent the mean of six or eight replicates ± 95% C.I.

Table 3.3: Toxicity endpoints (BEC_{10}, MDEC, NOEC, LOEC, EC_{50}, LC_{50}) calculated for *H. viridissima* and *M. mogurnda* exposed to Cu (µg L^{-1}) at three hardness levels, under constant alkalinity (4 mg L^{-1} as $CaCO_3$) and pH (6.0 ± 0.3) conditions, for 96 h.

Species	Hardness (mg L^{-1} as $CaCO_3$)	BEC_{10}	MDEC	NOEC	LOEC	Effect Concentration (95% C.I.)
Green hydra *(H. viridissima)*	6.6	0.8	1	0.9	1.9	4.6[a] (4.1-5.1)
	165	1.1	1.4	0.9	1.7	5.0[a] (4.5-5.5)
	330	0.8	0.9	0.9	1.8	5.5[a] (5.0-6.0)
Purple-spotted gudgeon *(M. mogurnda)*	6.6	6.4	8.8	11.4	11.8	13.0[b] (10.8-15.2)
	165	9.3	10.6	20	23.1	26.4[b] (23.1-29.7)
	330	5.7	6.9	19.7	24.4	23.4[b] (16.1-30.7)

[a] 50% Effect concentration (EC_{50})

[b] 50% Lethal concentration (LC_{50})

H. viridissima

At 6.6 mg L^{-1} hardness, the population growth of *H. viridissima* was reduced by 50% at 4.6 ± 0.5 µg L^{-1} Cu (Table 3.3). A 25-fold increase in water hardness (ie. from 6.6 to 165 mg L^{-1}) did not significantly ($P > 0.05$) affect the toxicity of Cu to *H. viridissima* (ie. overlapping 95% confidence intervals of the EC_{50} values; Table 3.3). Likewise, a 50-fold increase in water hardness (ie. from 6.6 to 330 mg L^{-1}) had no significant ($P > 0.05$) effect on Cu toxicity (ie. overlapping 95% confidence intervals of the EC_{50} values; Table 3.3).

The BEC_{10}, MDEC, NOEC and LOEC values are consistent with the trend reflected by the EC_{50} values (Table 3.3), supporting the lack of difference in Cu toxicity with increasing hardness. However, the BEC_{10} and MDEC values (1.1 and 1.4 µg L^{-1}, respectively) are slightly higher at 165 mg L^{-1} than at 330 mg L^{-1} hardness (0.8 and 0.9 µg L^{-1}, respectively).

M. mogurnda

In contrast to the results for *H. viridissima*, a 25-fold increase in water hardness significantly ($P \leq 0.05$) reduced the toxicity of Cu to *M. mogurnda* by two-fold (ie. an increase in the LC_{50} value from 13.0 to 26.4 µg L^{-1}; Table 3.3). Similarly, a 50-fold increase in water hardness also significantly ($P \leq 0.05$) reduced Cu toxicity by two-fold (ie. an increase in the LC_{50} value from 13.0 to 23.4 µg L^{-1}; Table 3.3). However, a two-fold increase in water hardness (ie. from 165 to 330 mg L^{-1} as $CaCO_3$) did not significantly ($P > 0.05$) affect Cu toxicity (ie. overlapping 95% confidence intervals of the LC_{50} values; Table 3.3).

The NOEC and LOEC values support the two-fold reduction in Cu toxicity between 6.6 and 330 mg L^{-1} hardness as suggested by the LC_{50} values (Table 3.3). It is interesting to note, that little difference was found between the NOEC and LOEC values at 6.6 mg L^{-1} (11.4 and 11.8 µg L^{-1}, respectively), and the LOEC at 330 mg L^{-1} hardness (24.4 µg L^{-1}) is greater than the respective LC_{50} (23.4 µg L^{-1}). Unlike the NOEC and LOEC values, the BEC_{10} and MDEC values do not reflect the trend observed by the LC_{50} values as these values at 330 mg L^{-1} hardness are lower than at 6.6 and 165 mg L^{-1} (Table 3.3).

Cu speciation

The predicted speciation (% distribution) of Cu in the test waters at pH 6.0 at the three hardness levels (6.6, 165 and 330 mg L^{-1} as $CaCO_3$) is given in Figure 3.4. No significant ($P > 0.05$) differences were found in the speciation of Cu between the three hardness levels. For example, the calculated activity of the free cupric ion (Cu^{2+}) for the *H. viridissima* EC_{50} and *M. mogurnda* LC_{50} values at each hardness are constant (95.8 – 96.8%; Appendix H).

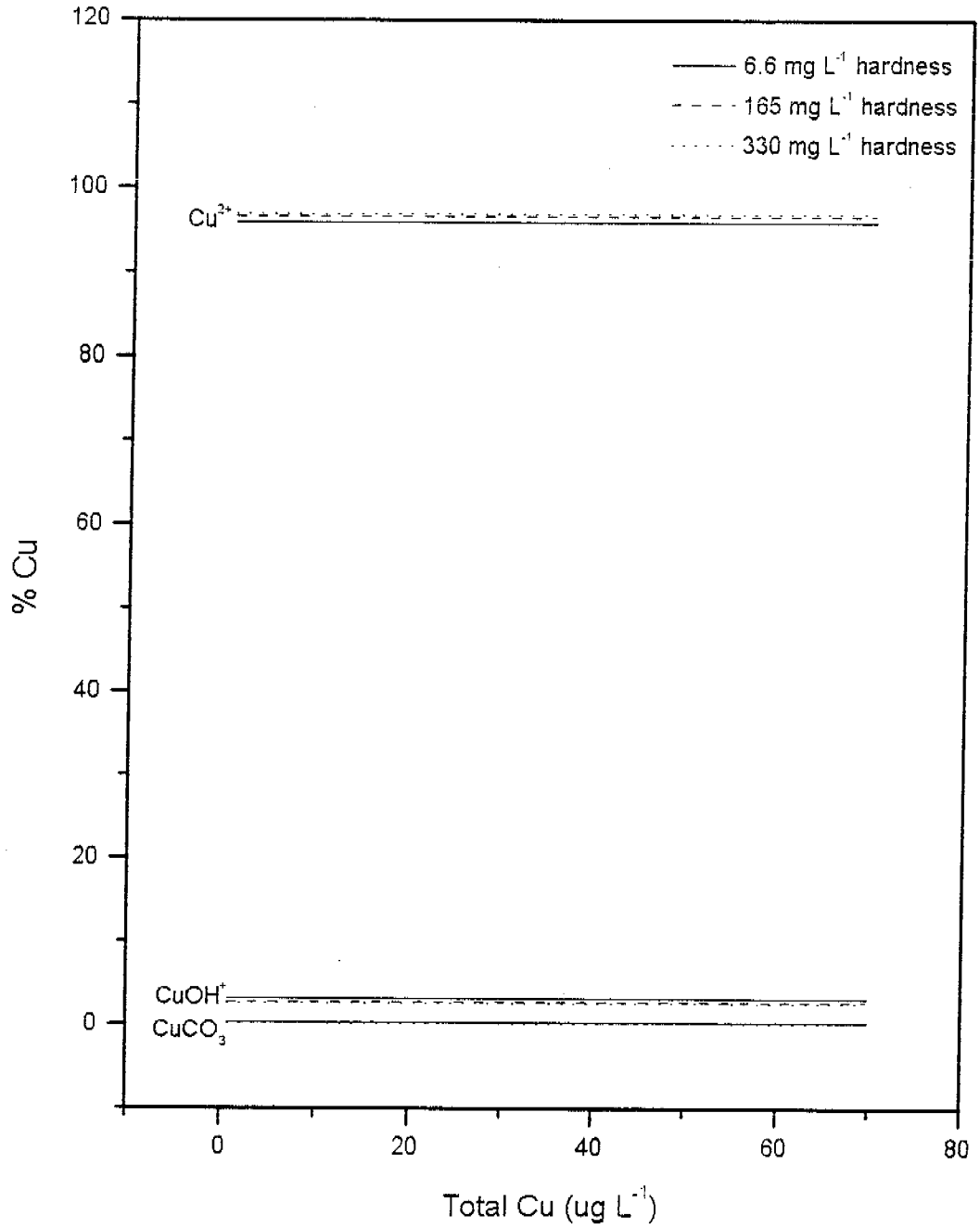

Figure 3.4: Predicted speciation (% distribution) of Cu in test water (pH 6.0) at three hardness levels (6.6, 165 and 330 mg L^{-1} as $CaCO_3$).

3.3.2 Influence of hardness on U toxicity

The concentration-response relationship for *H. viridissima* exposed to U at three hardness levels is shown in Figure 3.5. The concentration-response relationship for the first and second investigations of *M. mogurnda's* response to U at three hardness levels is shown in Figures 3.6 and 3.7, respectively. Summary data for each concentration-response curve is given in Appendix G, Table 3 and 4. The calculated BEC_{10}, MDEC, NOEC, LOEC, EC_{50} and LC_{50} values for *H. viridissima* and *M. mogurnda* exposed to U at three hardness levels (6.6, 165 and 330 mg L^{-1} as $CaCO_3$), are given in Table 3.4.

H. viridissima

Based on the EC_{50} values, a 25-fold increase in water hardness (ie. from 6.6 to 165 mg L^{-1} as $CaCO_3$) significantly ($P \leq 0.05$) reduced the toxicity of U to *H. viridissima* by 55% (ie. an increase in the EC_{50} value from 114 to 177 µg L^{-1}; Table 3.4). A 50-fold increase in water hardness (ie. from 6.6 to 330 mg L^{-1} as $CaCO_3$) significantly ($P \leq 0.05$) reduced the toxicity of U to *H. viridissima* by 92% (ie. an increase in the EC_{50} value from 114 to 219 µg L^{-1}; Table 3.4). A two-fold increase in water hardness (ie. from 165 to 330 mg L^{-1} as $CaCO_3$) significantly ($P \leq 0.05$) reduced the toxicity of U to *H. viridissima* by 24% (ie. an increase in the EC_{50} value from 177 to 219 µg L^{-1}; Table 3.4).

The trend observed for the EC_{50} is not consistent with BEC_{10} and MDEC values given in Table 3.4, due to differences between slopes of the concentration-response curves (Figure 3.5). The BEC_{10} and MDEC values at 165 mg L^{-1} hardness (81 and 90 µg L^{-1}, respectively), are higher than at 330 mg L^{-1} hardness (47 and 62 µg L^{-1}, respectively), due to the concentration-response curve of the former having a steeper slope. The NOEC and LOEC values at 165 mg L^{-1} hardness (150 and 162 µg L^{-1}, respectively), are also greater than at 330 mg L^{-1} hardness (62 and 87 µg L^{-1}, respectively).

M. mogurnda

The initial experimental series determining the effect of hardness on the toxicity of U to *M. mogurnda* produced a contrasting response to those performed using *H. viridissima*. A 25-fold increase in water hardness significantly ($P \leq 0.05$) increased the toxicity of U to *M. mogurnda* by 23% (ie. a decrease in the LC_{50} value from 1730 to 1335 µg L^{-1}; Table 3.4). Similarly, a 50-fold increase in water hardness significantly ($P \leq 0.05$) enhanced the toxicity of U to *M. mogurnda* by 26% (ie. a decrease in the LC_{50} value from 1730 to 1270 µg L^{-1}; Table 3.4). However, there was no difference in toxicity at the two higher hardness levels (ie. overlapping 95% confidence intervals of the LC_{50} values; Table 3.4). In contrast, the second investigation found that neither a 25-fold or 50-fold increase in water hardness had an effect on U toxicity to *M. mogurnda* (ie. overlapping 95% confidence intervals of the EC_{50} values; Table 3.4).

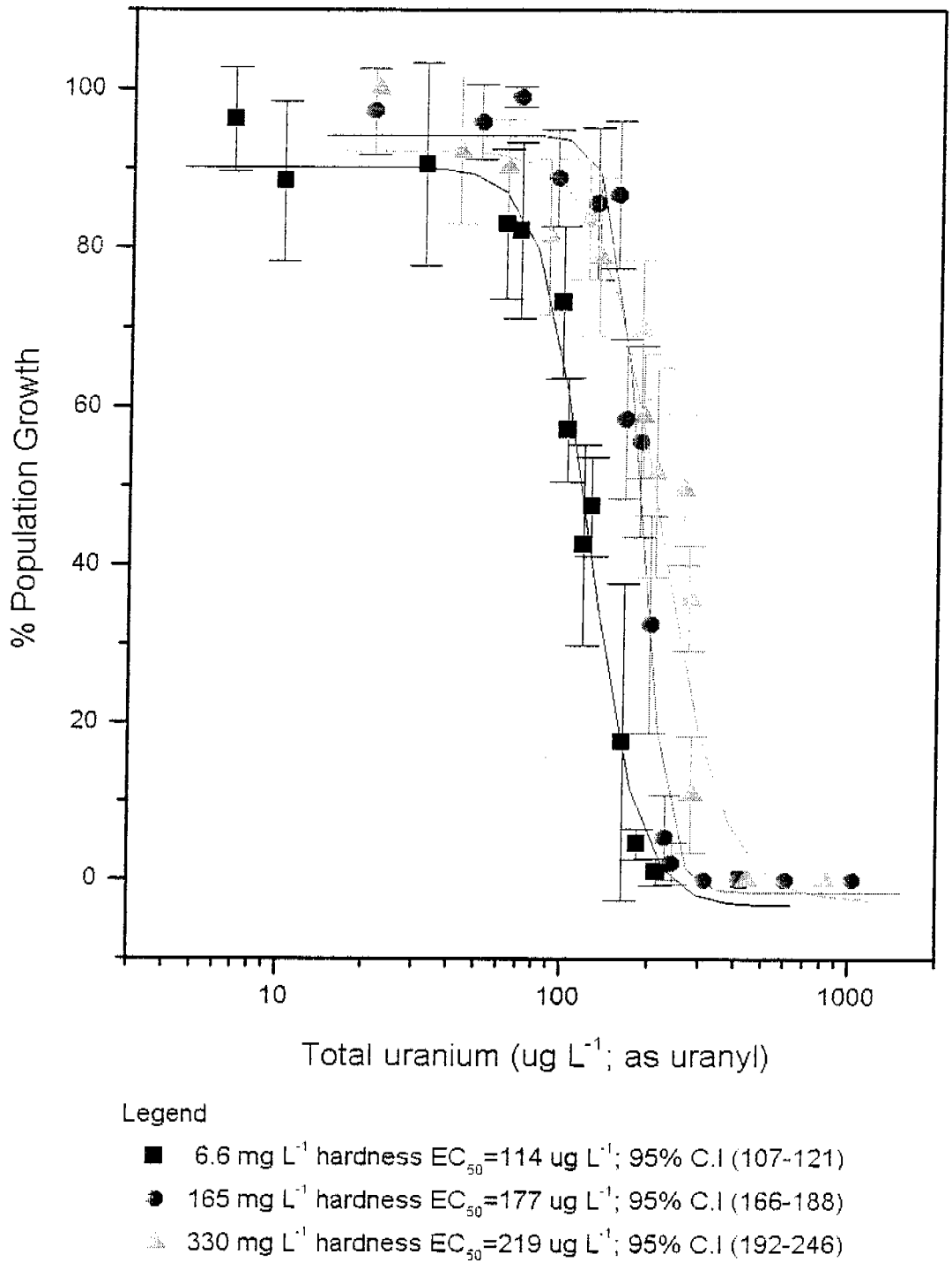

Figure 3.5: Population growth of *H. viridissima* exposed to U over 96 h at three hardness levels (6.6, 165 and 330 mg L^{-1}). Data points represent the mean of six or nine replicates ± 95% C.I.

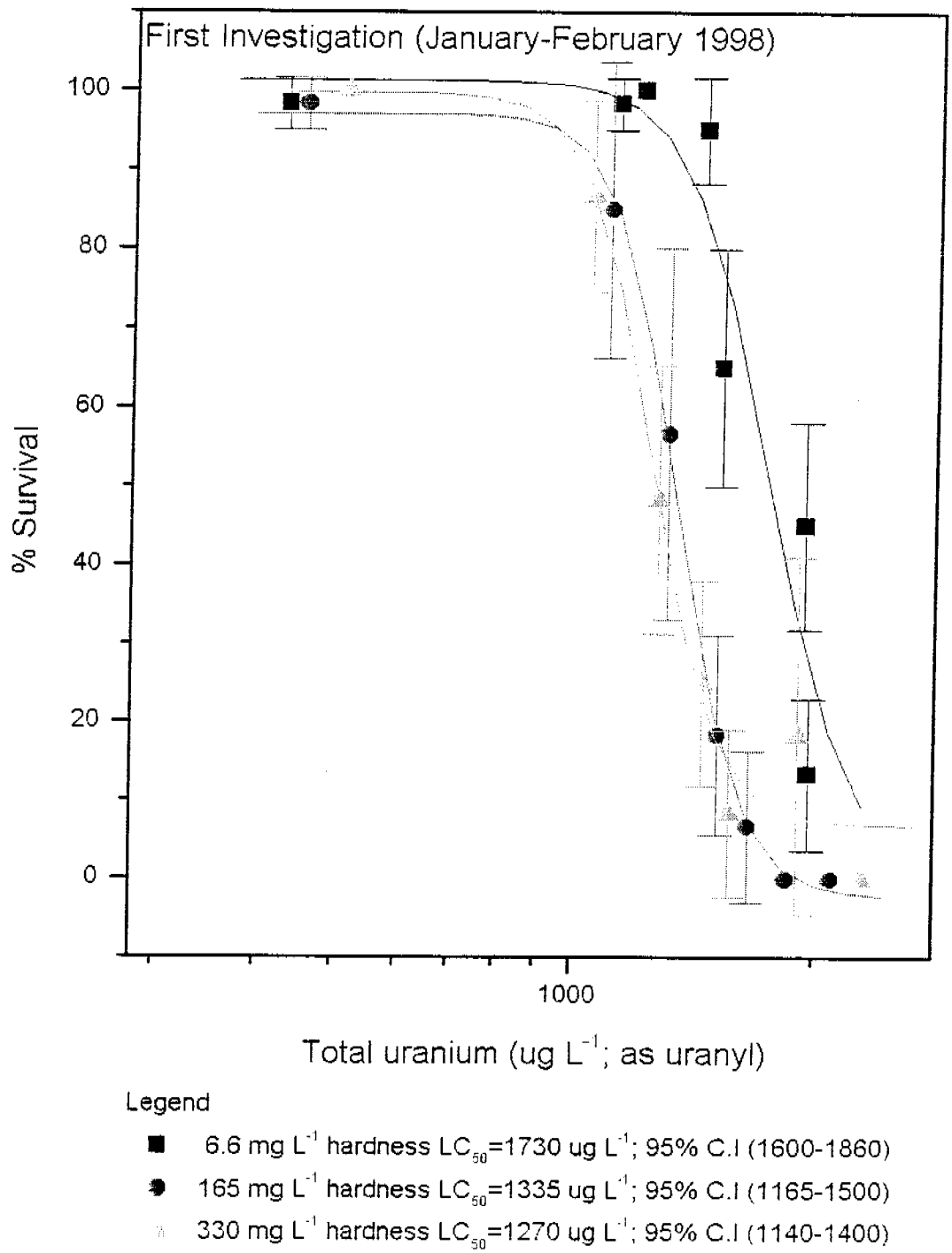

Figure 3.6: Survival of *M. mogurnda* exposed to U over 96 h at three hardness levels (6.6, 165 and 330 mg L^{-1}). Data points represent the mean of six or eight replicates ± 95% C.I.

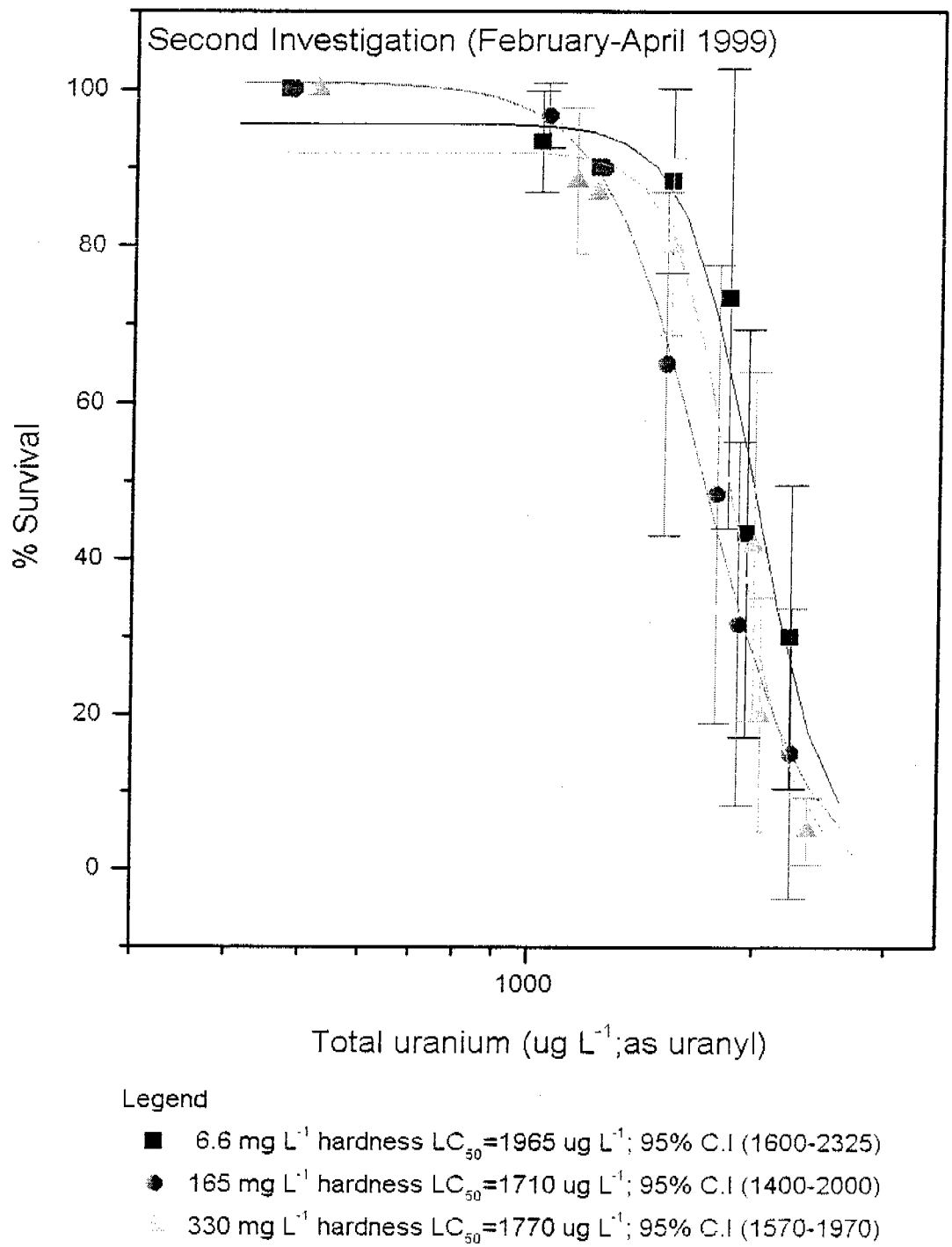

Figure 3.7: Survival of *M. mogurnda* exposed to U over 96 h at three hardness levels (6.6, 165 and 330 mg L^{-1}). Data points represent the mean of six or eight replicates ± 95% C.I.

Table 3.4: Toxicity endpoints (BEC_{10}, MDEC, NOEC, LOEC, EC_{50}, LC_{50}) calculated for *H. viridissima* and *M. mogurnda* exposed to U (µg L^{-1}) at three hardness levels, under constant alkalinity (4 mg L^{-1}) and pH (6.0 ± 0.3) conditions, for 96 h.

Species	Hardness (mg L^{-1} as $CaCO_3$)	BEC_{10}	MDEC	NOEC	LOEC	Effect Concentration (95% C.I)
Green hydra *(H. viridissima)*	6.6	14	32	32	62	114[a] (107-121)
	165	81	90	150	162	177[a] (166-188)
	330	47	62	62	87	219[a] (192-246)
Purple-spotted gudgeon *(M. mogurnda)*	**First investigation**					
	6.6	1410	1460	1450	1530	1730[b] (1600-1860)
	165	570	860	1100	1310	1335[b] (1165-1500)
	330	725	915	1050	1280	1270[b] (1140-1400)
	Second investigation					
	6.6	900	1220	1835	1950	1965[b] (1600-2325)
	165	1110	1240	1510	1770	1710[b] (1400-2000)
	330	860	1040	1530	1990	1770[b] (1570-1970)

[a] 50% Effect concentration (EC_{50})

[b] 50% Lethal concentration (LC_{50})

In the first investigation, the BEC_{10} and MDEC values at 165 mg L^{-1} hardness (570 and 860 µg L^{-1}, respectively), are slightly less than at 330 mg L^{-1} hardness (725 and 915 µg L^{-1}, respectively; Table 3.4), due to the steeper slope of the former concentration-response curve (Figure 3.6). Note also, that the LOEC at 330 mg L^{-1} hardness (1280 µg L^{-1}) is greater than the LC_{50} (1270 µg L^{-1}). Nonetheless, the BEC_{10}, MDEC, NOEC and LOEC values reflect a similar increase in U toxicity with increasing water hardness as that of the EC_{50} values (Table 3.4).

In the second investigation, the LOEC values for 165 and 330 mg L^{-1} hardness (1773 and 1989 µg L^{-1}, respectively) are higher than the corresponding LC_{50} (1706 and 1772 µg L^{-1}, respectively). In addition, the LOEC at 330 mg L^{-1} hardness (1989 µg L^{-1}) is greater than both the 6.6 mg L^{-1} hardness LOEC (1947 µg L^{-1}) and LC_{50} (1963 µg L^{-1}), opposing the LC_{50} trend. The trend within BEC_{10} and MDEC values is also inconsistent to that of the LC_{50} values (Table 3.4). The lack of consistency can be attributed to differences between slopes of the concentration-response curves (Figure 3.7). For example, the BEC_{10} and MDEC values at 165 mg L^{-1} hardness (1110 and 1240 µg L^{-1}, respectively), are greater than at 6.6 mg L^{-1} hardness (900 and 1220 µg L^{-1}, respectively), due to the steeper slope of the former concentration-response curve.

U speciation

The predicted speciation (% distribution) of U in the test waters at pH 6.0 at the three hardness levels (6.6, 165 and 330 mg L^{-1} as $CaCO_3$) is given in Figure 3.8. No significant ($P > 0.05$) differences were found in the speciation of U between the three hardness levels. For example, the calculated activity of the free uranyl ion (UO_2^{2+}) for the *H. viridissima* EC_{50} value at each hardness was constant (6.6 - 6.8%; Appendix H). Likewise, the calculated UO_2^{2+} activity for the *M. mogurnda* LC_{50} value at each hardness was constant (2.9 – 3.7%; Appendix H).

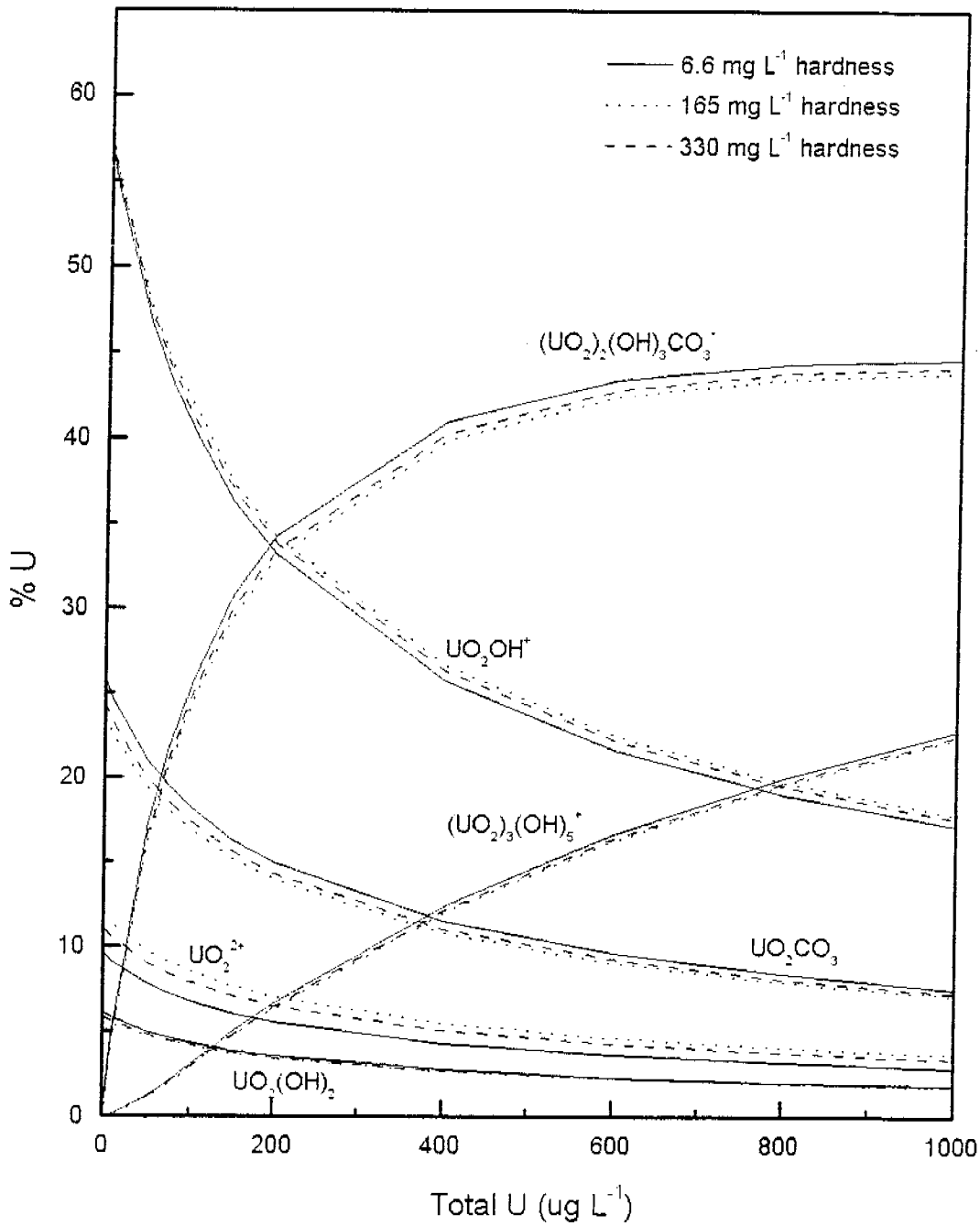

Figure 3.3: Predicted speciation (% distribution) of U in test water (pH 6.0) at three hardness levels (6.6,165 and 330 mg L^{-1} as $CaCO_3$). Uranyl species comprising < 2% total U are excluded for clarity.

3.4 Discussion

3.4.1 Influence of hardness on Cu toxicity

Copper toxicity to *H. viridissima* at 6.6 mg L^{-1} hardness was similar (ie. overlapping 95% confidence intervals of the EC_{50} values) to that reported by Markich and Camilleri (1997) under identical experimental conditions. The present study found the EC_{50} for *H. viridissima* to be 4.6 ± 0.5 µg L^{-1}, compared to 4.0 ± 0.25 µg L^{-1} reported by Markich and Camilleri (1997). The similarity between studies demonstrates the repeatability and validity of the bioassay procedure and the consistency of bioassay organism sensitivity over time. However, the present study found *M. mogurnda* to be significantly ($P \leq 0.05$) more sensitive to Cu at 6.6 mg L^{-1} hardness, compared to Markich and Camilleri's (1997) findings. The LC_{50} for *M. mogurnda* was 13.0 ± 2.2 µg L^{-1} in this study, compared to 22.1 ± 1.0 µg L^{-1} as reported by Markich and Camilleri (1997). Natural variability in sac-fry health and genetics (ie. parent stock) may largely explain the difference in metal sensitivity between studies, as discussed in Section 3.4.2.

Copper was less toxic to *M. mogurnda* at 6.6 mg L^{-1} than at 330 mg L^{-1} hardness, supporting previous observations in the literature that Cu toxicity decreases with increasing hardness. Erickson *et al.* (1996) found that Cu toxicity to fathead minnows (*Pimephales promelas*) decreased by 30% with a 20% increase in true water hardness. For rainbow trout (*Salmo gairdneri*; renamed *Oncorhynchus mykiss*), the toxicity of Cu was 3-fold greater at 12 mg L^{-1} Ca hardness than at 100 mg L^{-1} (Miller and Mackay, 1980). These authors concluded that Ca^{2+} and/or Mg^{2+} in solution offered some protection against toxic metal ions by competing for the same cellular binding sites. This mechanism of competition is not fully understood. It has been suggested, that as Ca^{2+} and Mg^{2+} increases, these ions displace Cu^{2+} from Ca channels at the cell surface, consequently decreasing metal uptake, and metal toxicity (Markich and Jeffree, 1994; Erickson *et al.*, 1996). Furthermore, Ca is thought to elicit a greater protective effect, relative to Mg, on the toxicity of trace metals to aquatic organisms (Carrol et al., 1979; Part *et al.*, 1985; Markich and Jeffree, 1994). For example, Part *et al.* (1985) reported 4-5-fold more Mg^{2+} was needed to obtain the same reduction in Cd transfer in rainbow trout (*Salmo gairdneri*; renamed *Oncorhynchus mykiss*) gills as that with Ca^{2+}. Partitioning the effects of Ca and Mg was not evaluated in this study.

A 50-fold increase in water hardness (ie. from 6.6 mg L^{-1} to 330 mg L^{-1} as $CaCO_3$) was shown to have no effect on the toxicity of Cu to *H. viridissima*. No previous data are available regarding the effects of true water hardness on Cu toxicity to freshwater hydra. However, Winner (1985) derived a similar conclusion when a four-fold increase in water hardness did not affect Cu toxicity to *Daphnia pulex*. Unfortunately, Winner's (1985) observation may have been influenced by the humic acid component of the experimental

media. Because increasing hardness did not ameliorate Cu toxicity, it seems the Ca^{2+} and Mg^{2+} competition mechanism for excluding Cu to *M. mogurnda* described above, is not amenable for *H. viridissima* and there is another mechanism in place.

The predicted percentage distribution of Cu in test waters (at pH 6.0) did not differ with increasing hardness, nor did the dominance of the free cupric ion (Cu^{2+}). This result eliminates the possibility that speciation influenced the concentration of soluble Cu or confounded the relationship found between hardness and Cu toxicity. Perhaps an experiment using a radio-tracer could provide evidence, which would distinguish between physiological, toxicological and metal speciation effects.

3.4.2 Influence of hardness on U toxicity

The response of *H. viridissima* to U at 6.6 mg L^{-1} hardness was consistent with the findings of Markich and Camilleri (1997) under identical experimental conditions. This study calculated the EC_{50} for *H. viridissima* to be 114 ± 7.0 µg L^{-1}, while Markich and Camilleri (1997) reported an EC_{50} value of 108 ± 6.0 µg L^{-1}. The similarity between studies demonstrates the consistency of the bioassay procedure and organism sensitivity over time. At 6.6 mg L^{-1} hardness, the sensitivity of *M. mogurnda* to U was found to be significantly ($P \leq 0.05$) less in this study compared to Markich and Camilleri's (1997) findings. The LC_{50} for *M. mogurnda* was 1730 ± 130 and 1965 ± 365 µg L^{-1} (ie. in the first and second investigations, respectively), compared to 1550 ± 34 µg L^{-1} as reported by Markich and Camilleri (1997). Again, natural variability in sac-fry health and genetics (ie. parent stock) may largely explain the difference in metal sensitivity between tests, as discussed below.

Based on 96 h EC_{50} values, the toxicity of U to *H. viridissima* decreased two-fold with a 50-fold increase in water hardness. Although previous studies derived a similar relationship for cladocera (Kennedy *et al.*, 1995; Barata *et al.*, 1998), they did not separate the effects of true water hardness (ie. Ca and Mg concentration) from alkalinity (ie. carbonate concentration) and/or pH. The result found in this study may be explained by the working hypothesis that Ca^{2+} and Mg^{2+} ions compete with the free metal ion (UO_2^{2+}) for binding sites to decrease metal (U) toxicity (Markich and Jeffree, 1994). Previous studies (Markich and Jeffree 1994, Issa *et al.* 1995, Erickson *et al.* 1996) have confirmed this hypothesis for other species of freshwater organisms (bivalves, fish and crustaceans) with trace metals (Cd, Cu, Mn, Pb and Zn).

The initial investigation found U to be 23% less toxic to *M. mogurnda* at 6.6 mg L^{-1} than at 165 mg L^{-1} hardness, suggesting U toxicity is somehow enhanced by the presence of Ca^{2+} and Mg^{2+}. Upon reinvestigating this result, hardness was found to have no affect on U toxicity to *M. mogurnda*. The difference between investigations of hardness effects on U

toxicity may be attributed, at least in part, to variability in sac-fry health and genetics. Sac-fry sensitivity to both Cu and U was shown to differ between the present study and Markich and Camilleri (1997). Such an observation suggests the protocol used in this study is neither robust nor repeatable when the physico-chemical parameters of the synthetic diluent water are manipulated.

As hardness does not appear to protect *M. mogurnda* from U toxicity, it is suggested another mechanism other than Ca^{2+} and Mg^{2+} competition is acting. The gills of freshwater fish are the primary uptake sites of Ca from surrounding water (Flik and Verbost, 1994). Trace metals appear to be taken up at the same sites as Ca, as indicated by data showing that Ca^{2+} competitively inhibits Al^{3+} (Verbost *et al.*, 1992) and Zn^{2+} (Hogstrand *et al.*, 1994). Interestingly, Part *et al.* (1985) found the retention of Cd by the gills of rainbow trout (*Salmo gairdneri*; renamed *Oncorhynchus mykiss*) to be unaffected by the external Ca^{2+} and Mg^{2+} concentration, concluding that no significant competition for binding sites occurred. Perhaps the system for Ca absorption in fish is also unable to retain U, preventing the Ca-Mg competition mechanism offering protection.

The predicted speciation (% distribution) of U in the test waters did not differ with increasing hardness. This result provides evidence that speciation did not effect the concentration of soluble U or confounded the relationship found between hardness and U toxicity. As suggested for Cu, further research involving an experiment using a radio-tracer could help distinguish between physiological, toxicological and metal speciation effects.

3.5 Conclusions

The effect of increasing water hardness was variable, depending on the metal and test organism investigated. It was found that a 50-fold increase in hardness had no effect on Cu toxicity to *H. viridissima*, but decreased U toxicity by approximately 2-fold. The opposite was observed for *M. mogurnda*, where increased hardness resulted in a 2-fold decrease in the toxicity of Cu, while it had no effect (ie. in the second investigation) on U toxicity. The observed toxicity effects of hardness occurred without any change in the speciation of Cu or U. The reduction in U toxicity to *H. viridissima* and Cu toxicity to *M. mogurnda* with increasing hardness may be explained by Ca-Mg competition mechanism, where the Ca and Mg ions compete with Cu/U ions for binding sites at the cell surface (Markich and Jeffree, 1994; Erickson *et al.*, 1996). However, the competition mechanism in not amenable where Cu toxicity to *H. viridissima* or U toxicity to *M. mogurnda* was not reduced, suggesting there is another mechanism in place.

4 Effect of alkalinity on the toxicity of Cu and U

4.1 Rationale

Many authors have described alkalinity to be an influential factor on metal toxicity. In attempting to define the effects of alkalinity (ie. carbonate concentration), several of these authors confounded their results with the effects produced by hardness (ie. Ca and Mg concentration) and pH (eg. Cu: Howarth and Sprague, 1978; U: Parkhurst *et al.*, 1984; Barata *et al.*, 1998). Those studies that manipulated the carbonate concentration independently of the Ca and/or Mg concentration, and pH, found increasing alkalinity to reduce the bioavailability and toxicity of Cu (Andrew *et al.*, 1977; Miller and Mackay, 1980; Laurén and McDonald, 1986; Daly *et al.*, 1990a). Similarly, alkalinity has been found to attenuate the adverse effects of U toxicity to a freshwater bivalve (*Velesunio angasi*), under constant water hardness and pH conditions (Markich *et al.*, 1996). Hardness and alkalinity effects need to be separated as the two variables affect metal toxicity differently, as described previously (Refer to Chapter 3).

Thus, one objective of this study was to isolate and assess the effects of alkalinity, at constant hardness and pH, on the toxicity of Cu and U to *H. viridissima* (Green hydra, population growth) and *M. mogurnda* (Purple-spotted gudgeon, sac-fry survival).

4.2 Methodology

Toxicity testing materials and procedure are detailed in Chapter 2. Only specific modifications made to these standard procedures are mentioned here.

4.2.1 Selection of alkalinity levels

Regional water quality information was gathered from Northern Territory Water Resources and the Environmental Research Institute of the Supervising Scientist to determine a relevant range of alkalinity for tropical Australian freshwater systems (Figure 3.1; Table 3.1). Three levels of alkalinity were selected – 4.0, 102 and 205 mg L^{-1} $CaCO_3$. The baseline alkalinity of 4 mg L^{-1} $CaCO_3$ represents the mean alkalinity of Magela Creek water (Refer to Figure 3.1). The rationale for using Magela Creek water as a baseline reference in this study is outlined in Appendix B.1.4. The other alkalinity levels were calculated to compliment the hardness levels studied (ie. 165 and 330 mg L^{-1} $CaCO_3$) and represent tropical Australian waters (Refer to Figure 3.1; Table 3.1).

4.2.2 Isolating alkalinity effects

Alkalinity was manipulating using $NaHCO_3$, while $Ca(NO_3)_2$ and $Mg(NO_3)_2$ continued to be added to the synthetic diluent water as detailed in Chapter 3. The rationale was that the difference in toxicity with the addition of carbonate to test waters at known hardness could be attributed as alkalinity effects. All other physico-chemical parameters were held constant (ie. pH 6.0 ± 0.3 and conductivity within 10% error, over 24 h).

Solutions containing the corresponding alkalinity and hardness levels were prepared, and examined for the formation of precipitants. A white precipitant formed at an alkalinity of 205mg L^{-1} as $CaCO_3$ after 72 h, while the other solutions appeared free of precipitants.

A preliminary test was conducted to ensure the pH, conductivity, dissolved oxygen (DO_2) and alkalinity of the test solutions remained within an acceptable range over 24 h. Physico-chemical parameters were measured as per Section 2.4. It was observed that the pH deviated beyond the acceptable range of 6.0 ± 0.3. This can be explained by the direct logarithmic relationship between pH and alkalinity, where the pH increases with the addition of carbonate and when the pH was lowered the carbonate is converted to CO_2 (Stumm and Mogan, 1981). A few techniques were examined to stabilise pH, and these are detailed in Appendix E. As a result of these investigations it was obvious a biological buffer was needed to maintain pH so that the effects of alkalinity would not be confounded. While biological buffers have proved successful in controlling pH in experimental systems (Stauber *et al.*, 1994; Franklin *et al.*, 1998), caution must be exercised as they have been shown to complex metals (Good *et al.*, 1966), and thus alter metal toxicity (Lage *et al.*, 1996). Consequently, the use of buffers in metal toxicity tests is generally avoided. MES (2-morpholinoethanesulfonic acid) biological buffer appeared suitable for this study as its pKa at 20°C is 6.15 (Good *et al.*, 1966), which is ideal for stabilising pH in the range of 6.0 ± 0.3. The suitability of MES was assessed as detailed below.

4.2.3 Incorporation of MES biological buffer into toxicity protocols

A series of tests were conducted to determine the concentration at which MES buffer maintained pH without having adverse effects on or altering the toxicity of Cu and U to the test organisms. Three buffer concentrations were selected - 2.5, 5.0 and 10 mM – based on Franklin *et al.* (1998). Each buffer concentration was added to each of the selected alkalinity solutions. In the presence of 2.5 mM buffer, the pH of each treatment increased by 2.0 units over 24 h. The 10 mM buffer maintained the pH of all treatments within 6.0 ± 0.3, but reduced the population growth of *H. viridissima* by 12-33% compared to growth measured in non-buffered treatments. The 5 mM buffer maintained the pH of 4 mg L^{-1} and 102 mg L^{-1} alkalinity solutions within 6.0 ± 0.3, but the pH of 205 mg L^{-1} solution increased by 2.0 units

over 24 h. *Mogurnda mogurnda* sac-fry survival showed no observed effect when exposed to 5 mM buffer solution. However, *H. viridissima* population growth decreased by 20% compared to growth in non-buffered treatments. Subsequently, a MES concentration of 4 mM was found to maintain the pH of 4 mg L^{-1} and 102 mg L^{-1} alkalinity solutions within 6.0 ± 0.3. In addition, *H. viridissima* population growth and *M. mogurnda* survival in buffered treatments, was similar to growth in non-buffered treatments. The alkalinity level of 205 mg L^{-1} $CaCO_3$ was excluded from this study as the 4 mM MES was unable to maintain solution pH within an acceptable range, and MES concentrations > 4mM reduced *H.viridissima* population growth. During the 4 mM buffer trial, the effects of $NaHCO_3$ and conductivity were also examined and found to have no effect on either *H. viridissima* or *M. mogurnda* control responses.

Tests were conducted to investigate the effect of 4mM MES buffer on the toxicity of Cu and U to *H. viridissima* and *M. mogurnda*. A range of metal concentrations was selected based on the results reported in Chapter 3. Two tests were run in parallel – one containing the buffer solution and one without. The 4 mM buffer solution did not significantly ($P \geq 0.05$) affect the toxicity of either Cu or U to *H. viridissima* (ie. overlapping 95% confidence intervals of the EC_{50} values; Table 4.1). These results suggest 4mM MES buffer had no effect on Cu and U toxicity to *H. viridissima*. In contrast, *M. mogurnda* sac-fry showed a decrease in sensitivity to Cu with the incorporation of MES buffer at a concentration of 4 mM. At 110 ppb Cu, 3.3% survival was recorded in non-buffered water while 80% survival was recorded in buffered water, indicating that Cu toxicity to *M.mogurnda* was reduced by 4mM MES buffer. For this reason, the effect of alkalinity on the toxicity of Cu and U to *M. mogurnda* was not investigated. Subsequently, this study focused on the effect of alkalinity on Cu and U toxicity to *H. viridissima*.

Table 4.1: Population growth of *H. viridissima* exposed Cu and U in the presence and absence of 4 mM MES biological buffer.

Metal	50% Effect concentration (95% C.I.)	
	Buffer absent	Buffer present
Cu	8.1 (7.8-8.4)	6.7 (4.5-8.9)
U	230 (198-267)	210 (194-225)

4.3 Results

This study was designed to assess the effects of alkalinity (carbonate concentration) on the toxicity of Cu and U to *H. viridissima*, at constant water hardness (165 mg L^{-1} as $CaCO_3$) and pH (6.0 ± 0.3). Raw data for each test-series of a given metal-organism exposure are provided in Appendix F, Tables 5 and 6.

4.3.1 Influence of alkalinity on Cu toxicity to *H. viridissima*

The concentration-response relationship for *H. viridissima* exposed to Cu at two alkalinity levels is shown in Figure 4.1. Summary data for the concentration-response curve is given in Appendix G, Table 5. The calculated BEC_{10}, MDEC, NOEC, LOEC, and EC_{50} values for *H. viridissima* exposed to Cu at two alkalinity levels (4 and 102 mg L^{-1} as $CaCO_3$), are given in Table 4.2.

Table 4.2: Toxicity endpoints (BEC_{10}, MDEC, NOEC, LOEC, EC_{50}) calculated for *H. viridissima* exposed to Cu (µg L^{-1}) at two alkalinity levels, under constant hardness (165 mg L^{-1} as $CaCO_3$) and pH (6.0 ± 0.3) conditions, for 96 h.

Alkalinity (mg L^{-1} as $CaCO_3$)	BEC_{10}	MDEC	NOEC	LOEC	EC_{50} (95% C.I.)
4	1.1	1.4	0.9	1.7	5.0 (4.5-5.5)
102	1.2	1.4	0.7	1.8	6.0 (5.5-6.5)

A 25-fold increase in alkalinity (ie. from 4.0 to 102 mg L^{-1} as $CaCO_3$), at a hardness of 165 mg L^{-1} as $CaCO_3$ did not significantly ($P > 0.05$) affect the toxicity of Cu to *H. viridissima* (ie. overlapping 95% confidence intervals of the EC_{50} values; Table 4.2). The trend observed for the EC_{50} values is consistent with the BEC_{10}, MDEC, NOEC and LOEC values given in Table 4.2, such that all endpoints suggest there is no difference in Cu toxicity with a 25-fold increase in alkalinity.

Cu speciation

The predicted speciation (% distribution) of Cu in the test waters at pH 6.0 at the two alkalinity levels (ie. 4.0 and 102 mg L^{-1} as $CaCO_3$) is given in Figure 4.2. Copper(II) was found to be the dominant species in both alkalinity solutions. In absolute terms, the free cupric ion (Cu^{2+}) was 5% more available in the 4.0 mg L^{-1} alkalinity solution than the 102 mg L^{-1} alkalinity solution (Appendix H, Table 3).

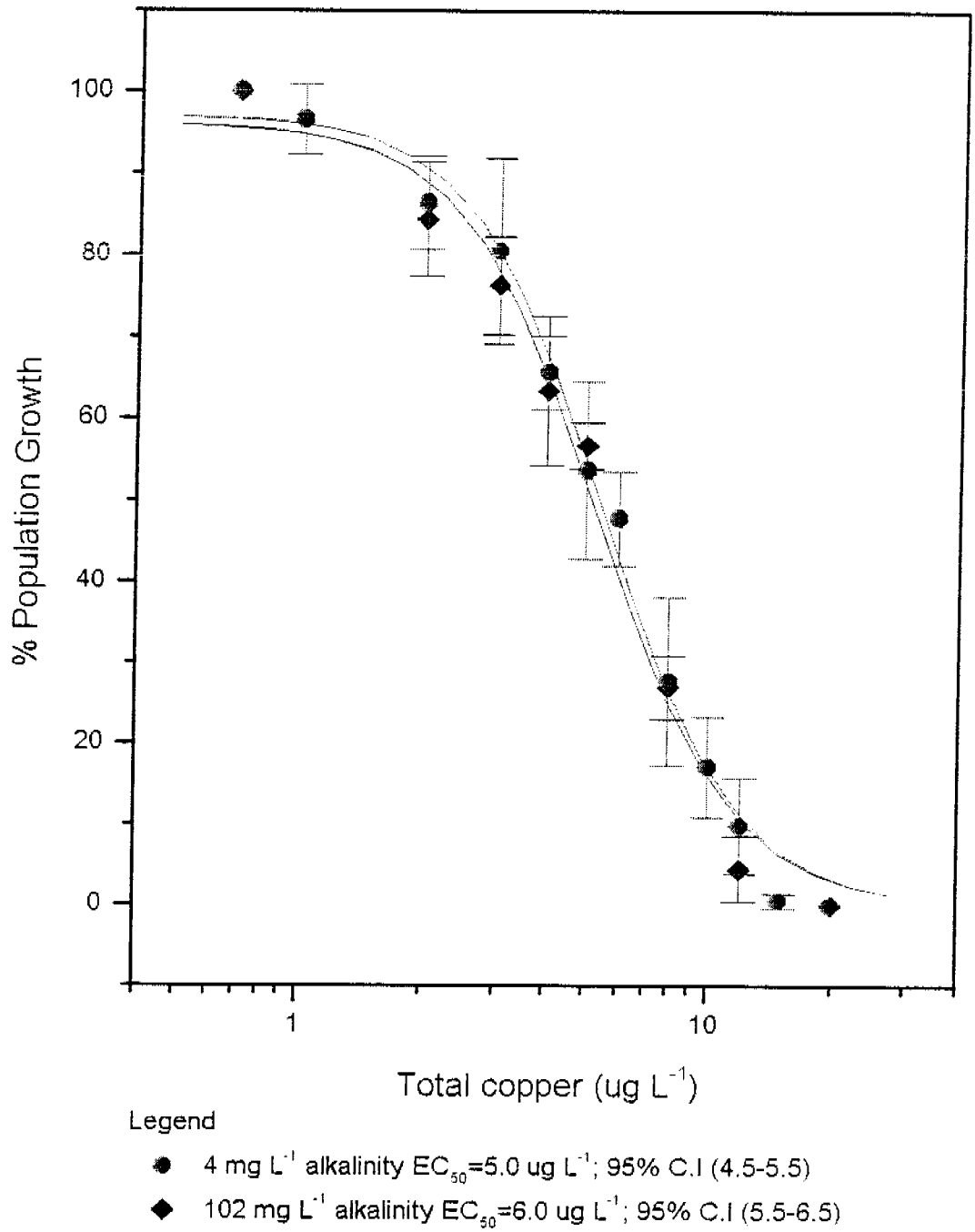

Figure 4.1: Population growth of *H. viridissima* exposed to Cu over 96 h at two alkalinity levels (4 and 102 mg L^{-1}). Data points represent the mean of six or nine replicates ± 95% C.I.

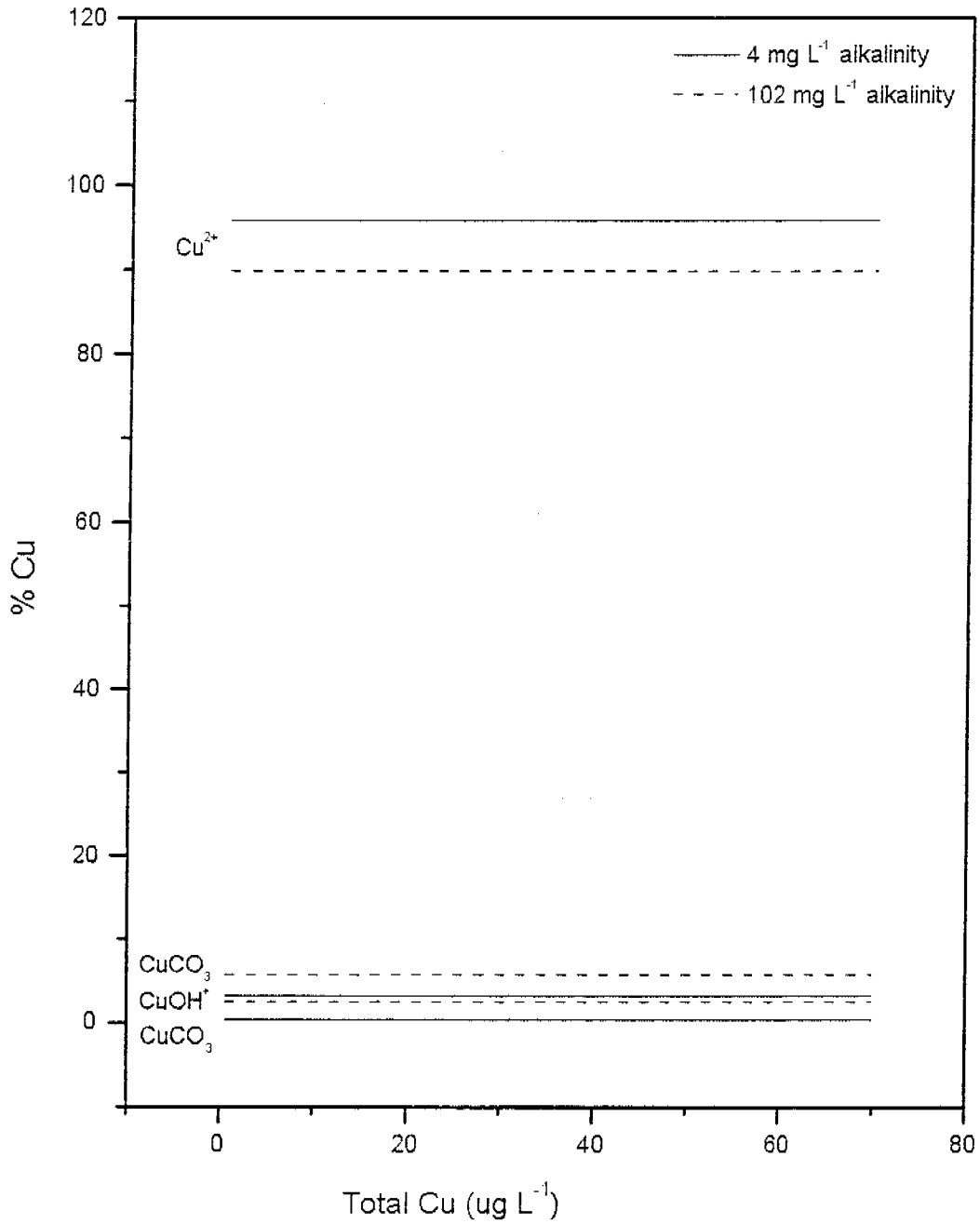

Figure 4.2: Predicted speciation (% distribution) of Cu in test water (pH 6.0) at two alkalinity levels (4 and 102 mg L^{-1} as $CaCO_3$).

4.3.2 Influence of alkalinity on U toxicity to *H. viridissima*

The concentration-response relationship for *H. viridissima* exposed to U is shown in Figure 4.3. Summary data for the concentration-response curve is given in Appendix G, Table 6. The calculated BEC_{10}, MDEC, NOEC, LOEC, EC_{50} values for *H. viridissima* exposed to U at two alkalinity levels (ie. 4.0 and 102 mg L^{-1} as $CaCO_3$), are given in Table 4.3.

Table 4.3: Toxicity endpoints (BEC_{10}, MDEC, NOEC, LOEC, EC_{50}) calculated for *H. viridissima* exposed to U (µg L^{-1}) at two alkalinity levels, under constant hardness (165 mg L^{-1} as $CaCO_3$) and pH (6.0 ± 0.3) conditions, for 96 h.

Alkalinity (mg L^{-1} as $CaCO_3$)	BEC_{10}	MDEC	NOEC	LOEC	EC_{50} (95% C.I.)
4	81	90	150	162	177 (166-188)
102	25	42	130	171	171 (150-192)

Based on EC_{50} values, a 25-fold increase in alkalinity (ie. from 4.0 to 102 mg L^{-1} as $CaCO_3$), at a hardness of 165 mg L^{-1} as $CaCO_3$ did not significantly ($P > 0.05$) affect the toxicity of U to *H. viridissima* (ie. overlapping 95% confidence intervals of the EC_{50} values; Table 4.3).

The slopes of the two alkalinity concentration-response curves differ, thus precluding a reasonable comparison of the BEC_{10} and MDEC values (Figure 4.3). The BEC_{10} and MDEC at 102 mg L^{-1} alkalinity (25 and 42 µg L^{-1}, respectively), are lower than at 4.0 mg L^{-1} alkalinity (81 and 90 µg L^{-1}, respectively) (Table 4.3), due to the concentration-response curve of the latter having a steeper slope. Note also, that the LOEC at an alkalinity of 102 mg L^{-1} as $CaCO_3$ is equivalent to the EC_{50} (Table 4.3).

U speciation

The predicted speciation (% distribution) of U in the test waters at pH 6.0 at two alkalinity levels (4.0 and 102 mg L^{-1} as $CaCO_3$) is given in Figure 4.4. A 25-fold increase in alkalinity (ie. carbonate concentration) altered the calculated U speciation through inorganic complexation. At ~175 µg L^{-1} U (ie. EC_{50} value; Appendix H, Table 4), the percentages of UO_2CO_3 increased by a factor of four at 102 mg L^{-1} as $CaCO_3$ (compared to the baseline alkalinity of 4.0 mg L^{-1} as $CaCO_3$), whilst the percentages of UO_2^{2+} and UO_2OH^+ decreased by a factor of six. The polmeric U species, $(UO_2)_3(OH)_3CO_3^-$, was also calculated to decrease by a factor of two. The increased alkalinity also substantially increased the percentage of $UO_2(CO_3)_2^{2-}$ from < 1 to 20%.

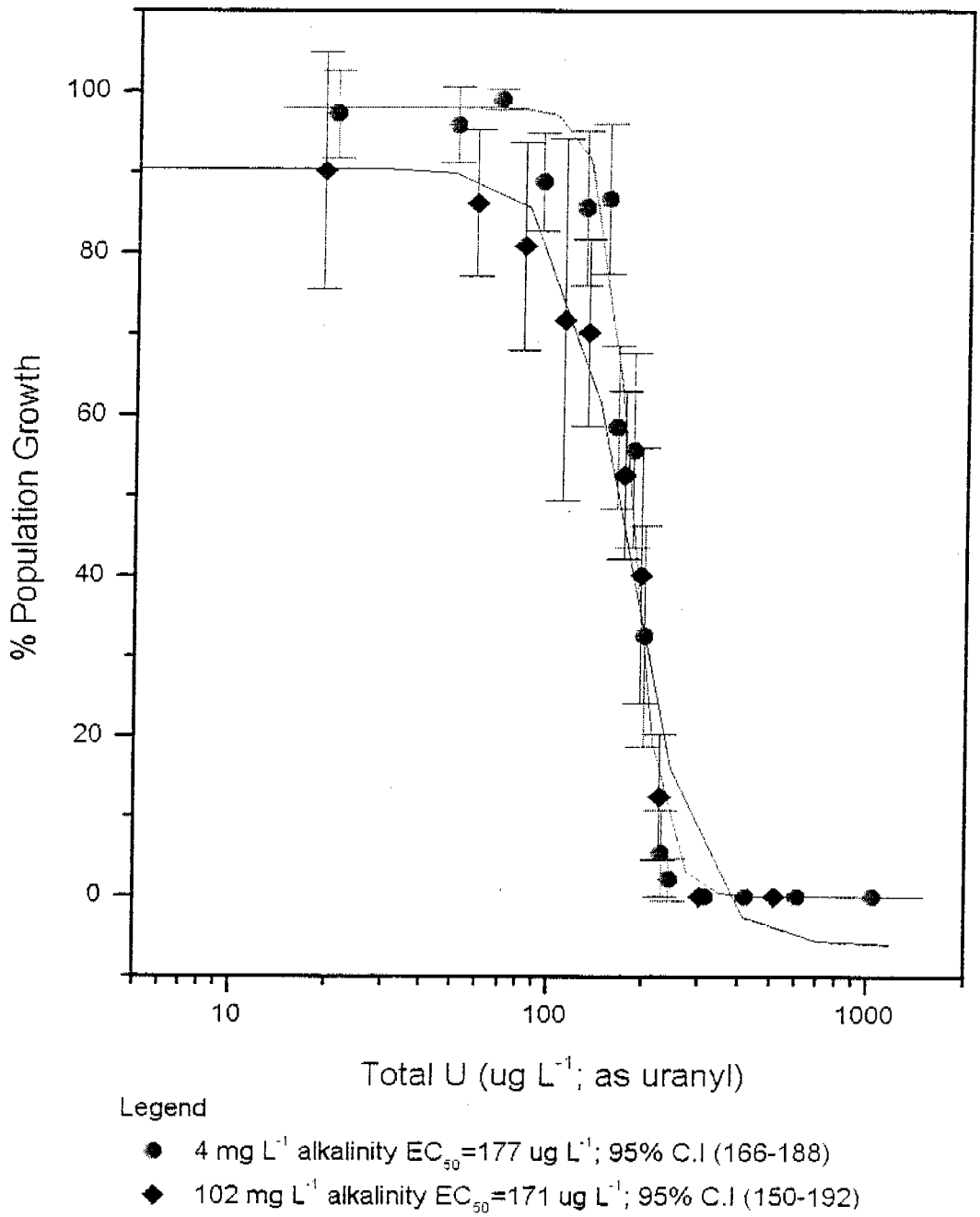

Figure 4.3: Population growth of *H. viridissima* exposed to U over 96 h at two alkalinity levels (4 and 102 mg L^{-1}). Data points represent the mean of six or nine replicates ± 95% C.I.

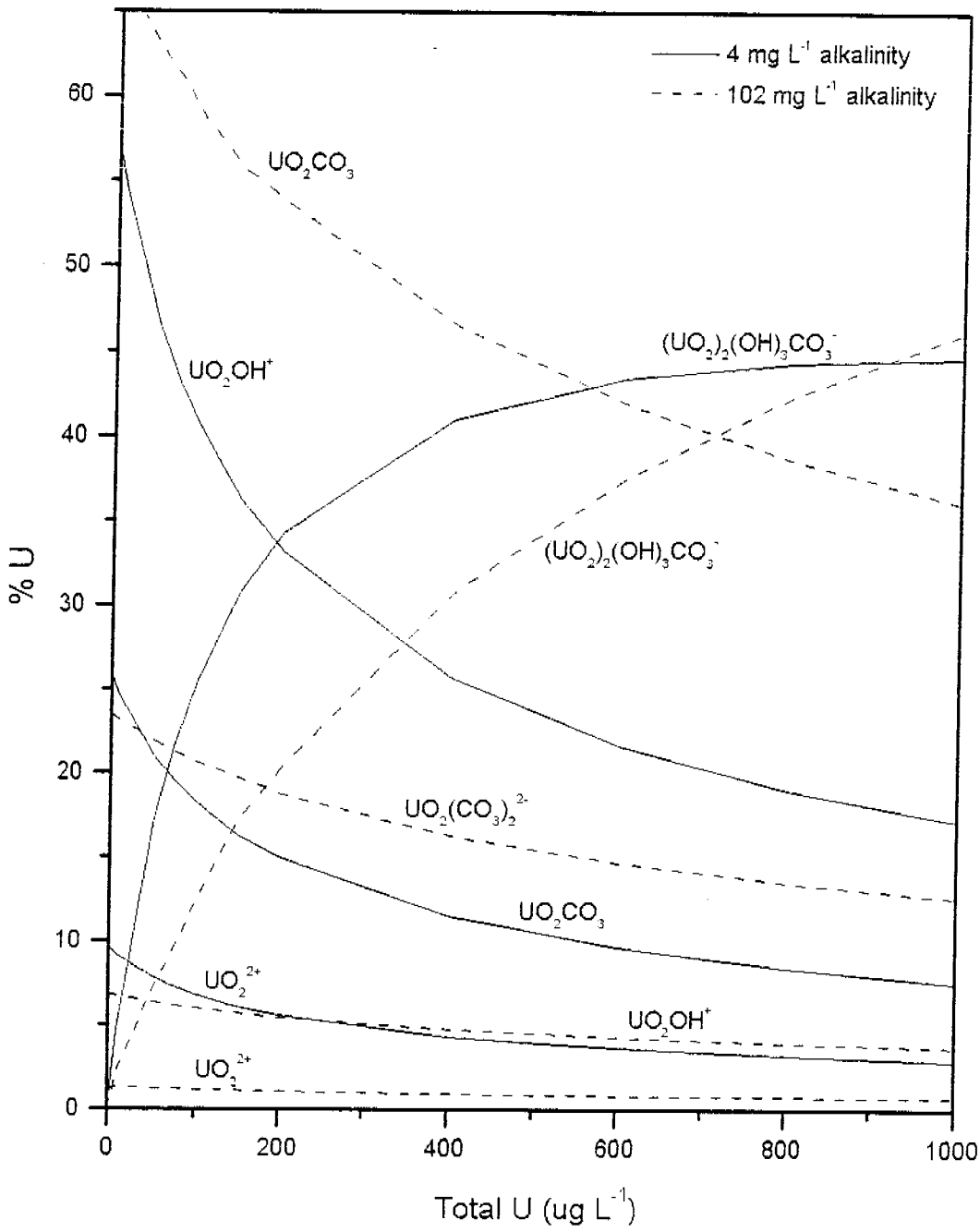

Figure 4.4: Predicted speciation (% distribution) of U in test water (pH 6.0) at two alkalinity levels (4 and 102 mg L^{-1} as $CaCO_3$). Uranyl species comprising < 2% total U are excluded for clarity.

4.4 Discussion

4.4.1 Influence of alkalinity on Cu toxicity to *H. viridissima*

The toxicity of Cu to *H. viridissima* did not differ with an increase in alkalinity from 4.0 mg L^{-1} to 102 mg L^{-1} (as $CaCO_3$), contrary to previous reports in the literature. Daly *et al.* (1990b) reported Cu toxicity to the Australian freshwater shrimp, *Paratya australiensis*, decreased in solutions of increasing alkalinity. Likewise, Andrew *et al.* (1977) found that the sensitivity of *D. magna* to Cu decreased when the alkalinity of the test solution was increased. These authors attributed the formation of copper-carbonate complexes to the reduction of Cu^{2+} activity, subsequently decreasing the uptake and toxicity of Cu.

The percent distribution of Cu^{2+} in the test waters differed by 5% between 4.0 mg L^{-1} and 102 mg L^{-1} alkalinity, providing supportive evidence for the fact that alkalinity did not affect Cu toxicity to *H. viridissima* at the pH and increased alkalinity used in this experiment. More specifically, the addition of carbonate ions did not alter the proportional relationship between Cu species, therefore not influencing the toxicity of Cu.

4.4.2 Influence of alkalinity on U toxicity to *H. viridissima*

The present study found that a 25-fold increase in alkalinity did not affect the toxicity of U to *H. viridissima*, at pH 6.0. This contrasts with the results of Markich *et al.* (1996) who found a 5-fold increase in alkalinity reduced U toxicity by 20% in the freshwater bivalve, *Velesunio angasi*, at pH 5. The different observations made in these studies may be attributed to the use of different pH levels and/or use of different test organisms. Evidence of such reasoning is provided by Markich *et al.* (1996), who found U toxicity decreased with increasing pH from 5.0 to 6.0, while the relative proportions of UO_2^{2+} and UO_2OH^+ declined, and several uranyl carbonates and hydroxides increased.

In contrast to the effects of increased Ca and Mg concentration, the increased alkalinity (ie. bicarbonate concentration) altered the calculated U speciation through inorganic complexation. Despite the changes in the calculated U speciation, there was no change in U toxicity. The absolute percent change in UO_2^{2+} from 6 to 1%) is minimal, given the errors associated with the selected stability constants used in the calculations. Therefore, according to the FIAM, which interprets that the toxic effect of U to *H. viridissima* is governed by UO_2^{2+}, then a minimal change in U toxicity would be expected. The FIAM could be further tested by creating a larger absolute percentage difference between the calculated activity of UO_2^{2+}, by slightly reducing the pH of the test waters.

4.5 Conclusions

MES biological buffer (4mM at pH 6.0; alkalinity 4 and 102 mg L-1) was found to be a suitable and practical option of controlling the pH in the bioassay protocols used in this study. Although the buffer enhanced *M. mogurnda* survival in elevated levels of Cu, the buffer did not affect *H. viridissima* pop growth or toxicity of Cu and U. A 25-fold increase in alkalinity at constant hardness and pH. was found to have no effect on the toxicity of either Cu or U to *H. viridissima*. The toxicity effects of Cu occurred without any change in speciation. In contrast, U speciation was altered with increasing alkalinity through inorganic complexation, despite no change in U toxicity. These results indicate that carbonate alkalinity does not affect Cu and U toxicity under the experimental conditions of this study.

5 General discussion

Metal speciation and bioavailability in fresh surface waters may be influenced by a variety of physico-chemical variables, particularly water hardness, alkalinity, pH, natural organic matter and redox potential (Hamelink *et al.*, 1994; Markich *et al.*, 1997). Quantitative relationships (algorithms) have only been established to describe the reduction in the bioavailability of Cd, Cr(III), Cu, Ni, Pb and Zn as a function of increasing hardness. Such algorithms have been incorporated into the water quality guidelines of several countries for the protection of aquatic organisms (CCREM, 1991; US EPA, 1995; ANZECC and ARMCANZ, 1999).

Although several studies have found water hardness to reduce Cu (Gauss *et al.*, 1985; Belanger *et al.*, 1989) and U (Parkhurst *et al.*, 1984; Barata *et al.*, 1998) toxicity to freshwater biota, insufficient and/or inconsistent data have precluded an algorithm being established. These, and other, studies that have investigated the effects of water hardness on the toxicity of metals to freshwater biota have confounded the effects of true water hardness (ie. Ca and/or Mg concentration) with alkalinity (ie. carbonate concentration) and pH (ie. proton concentration), since water hardness is often positively correlated with alkalinity in natural waters (Stumm and Morgan, 1981). The relative contribution of hardness and alkalinity in reducing metal toxicity is of importance, as each variable affects toxicity differently. Hardness (ie. Ca and/or Mg) competitively inhibit the uptake, and hence, toxicity of trace metals at the cell membrane surface (Markich and Jeffree, 1994), while alkalinity (ie. carbonate) complexes with trace metals, reducing the concentration(s) of toxic metal species (ie. a change in metal speciation) (Hunt, 1987).

Several studies have reported that the toxicity and bioavailability of Cu is ameliorated with increasing water hardness (Miller and Mackay, 1980; Mierle, 1981; Horne and Dunson, 1995 and Erickson *et al.*, 1996) and alkalinity (Andrew *et al.*, 1977; Miller and Mackay, 1980; Laurén and McDonald, 1986; Daly *et al.*, 1990a), without confounding parameters. The present study provides the first data concerning the effects of true water hardness on U toxicity to freshwater biota, while only one other study (Markich *et al.*, 1996) has described the effects of alkalinity on the toxicity of U to a tropical freshwater organism.

In light of this information, the present study determined the individual effects of true water hardness and alkalinity on the 96 h toxicity of Cu and U to *H. viridissima* and *M. mogurnda*, at constant pH. Such data has provided a greater understanding of the relationship between water hardness and alkalinity, and hence, provided a greater predictive ability of metal toxicity and bioavailability in tropical Australian freshwater systems.

5.1 Comparative sensitivity of test organisms to Cu and U toxicity

At baseline hardness (ie. 6.6 mg L^{-1} $CaCO_3$) and alkalinity (ie. 4.0 mg L^{-1} $CaCO_3$), *H. viridissima* was found to be more sensitive to both Cu and U compared to *M. mogurnda* (Tables 5.1 and 5.2). However, the difference in organism sensitivity is not proportional for both metals. *H. viridissima* is approximately three-fold more sensitive to Cu than *M. mogurnda*, and about 16-fold more sensitive to U.

The relative sensitivity of *H. viridissima* and *M. mogurnda* to Cu and U can be compared with other tropical freshwater species. For comparative purposes, only toxicity data derived under similar experimental conditions (ie. softwater, slightly acidic, low alkalinity and conductivity) to this study are reviewed (Tables 5.1 and 5.2). For a tropical freshwater alga (*Chlorella sp.*), Franklin *et al.* (1998) reported a 72 h EC_{50} value for growth inhibition of 35 µg L^{-1} Cu at pH 5.7, and 1.5 µg L^{-1} Cu at pH 6.5. Investigations using the valve movement of a freshwater bivalve (*Velesunio angasi*) found a 48 h EC_{50} of 10 µg L^{-1} Cu (pH 6.0) (Markich *et al.*, 1996). The present study found a 96 h EC_{50} value of 4.6 µg L^{-1} Cu for *H. viridissima* and a 96 h LC_{50} value of 13 µg L^{-1} Cu for *M. mogurnda* (pH 6.0). Although these studies were conducted at different pH levels, the sensitivity of these species can still be compared, as Cu speciation is similar between pH 5.7 and 6.0 (Franklin *et al.*, 1998). Based on EC_{50} values, *H. viridissima* appears to be more sensitive to Cu than the bivalve and alga. Unfortunately, the 'sublethal' endpoint measuring the response of hydra, algae and bivalve to Cu is not directly comparable to the less sensitive 'lethal' endpoint measuring *M. mogurnda* survival (Hendriks, 1995).

Table 5.1: Comparative toxicity of Cu to Australian tropical freshwater biota.

Species	Endpoint	Cu toxicity (µg L^{-1})	Reference
Cnidaria (*H. viridissima*)	96 h EC_{50}	4.0 (pH 6.0)	Markich & Camilleri (1997)
		4.6 (pH 6.0)	This study
Mollusca (*V. angasi*)	48 h EC_{50}	10 (pH 6.0)	Markich (1998)
Alga (*Chlorella sp.*)	72 h EC_{50}	35 (pH 5.7)	Franklin *et al.* (1998)
		1.5 (pH 6.5)	
Chordata (*M. mogurnda*)	96 h LC_{50}	22.1[a] (pH 6.0)	Markich & Camilleri (1997)
		13[a] (pH 6.0)	This study

[a] LC_{50} values cannot be directly compared to the EC_{50} values in this table.

The sensitivity of these organisms to U revealed a similar trend (Table 5.2). Franklin *et al.* (1998) reported a 72 h EC_{50} value of 78 µg L^{-1} U at pH 5.7, and 44 µg L^{-1} U at pH 6.5 for the toxicity of U to the alga, *Chorella sp.* Assessment of the toxicity of U to the freshwater bivalve found a 48 h EC_{50} value of 254 µg L^{-1} U at pH 5.8 (Markich *et al.*, 1996). In the present study, a 96 h EC_{50} value of 114 µg L^{-1} U was reported for *H. viridissima*, while a 96 h EC_{50} value of 1730 and 1955 µg L^{-1} U was reported for *M. mogurnda*, in the first and second investigation respectively (pH 6.0). Despite different test durations used, comparison of the EC_{50} values suggest *H.viridissima* is less sensitive compared to the alga, but more than the bivalve. Although the acute response of *M. mogurnda* in this study cannot be directly compared to the chronic response of the other species listed in Table 5.1, the 7 d chronic response of *M. mogurnda* to U investigated by Holdway (1992), may provide a comparison. Holdway (1992) reported a NOEC and LOEC of 920 and 1780 µg L^{-1} U, respectively. These values are greater than the EC_{50} values for alga, hydra and bivalve, suggesting *M. mogurnda* could potentially be the least sensitive species in the suite of organisms listed in Table 5.2.

Table 5.2: Comparative toxicity of U to Australian tropical freshwater biota.

Species	Endpoint	U toxicity (µg L^{-1})	Reference
Alga (*Chlorella sp.*)	72 h EC_{50}	78 (pH 5.7)	Franklin *et al.* (1998)
		44 (pH 6.5)	
Cnidaria (*H. viridissima*)	96 h EC_{50}	95 (pH 6.0)	Markich & Camilleri (1997)
		114 (pH 6.0)	This study
Mollusca (*V. angasi*)	48 h EC_{50}	254 (pH 6.0)	Markich *et al.* (1996)
Chordata (*M. mogurnda*)	96 h LC_{50}	1550[c] (pH 6.0)	Markich & Camilleri (1997)
		1730[a,c] and	This study
		1965[b,c] (pH 6.0)	

[a] LC_{50} from first investigation.

[b] LC_{50} from second investigation.

[c] LC_{50} values cannot be directly compared to the EC_{50} values in this table.

It should be noted that the trends described above are based on a single species from each phylum. Consequently, the comparisons made may not be a true indication of the relative sensitivities when multiple species are compared. However, it does suggest *H.viridissima* and *M. mogurnda* represent a range of metal sensitivities among tropical freshwater biota, and are therefore good indicators of metal contamination in tropical Australian freshwaters.

5.2 Effect of hardness on Cu and U toxicity

Increasing the true hardness of the test water (ie. Ca and Mg concentration) had a variable effect, depending on the metal and test organism investigated. A 50-fold increase in hardness resulted in a 2-fold decrease in the toxicity of Cu to *M. mogurnda*, while it had no effect (ie. in the second investigation) on U toxicity. The opposite was observed for *H. viridissima*, where increased hardness had no effect on Cu toxicity, but decreased U toxicity by approximately 2-fold. The observed effects of hardness on toxicity occurred without any change in the speciation of Cu or U. Such evidence supports the hypothesis that the protective effect of increased Ca^{2+} and/or Mg^{2+} involves a biological mechanism (Bradley and Sprague, 1985; Part *et al.*, 1985; Markich and Jeffree, 1994). Markich and Jeffree (1994) found that some metals (ie. Pb, Cd, Mn and Co) are adsorbed as analogues of Ca from the aquatic medium, suggesting that Ca ions compete with the free ionic species for binding sites at the membrane surface. This biological mechanism may explain the effect hardness had on reducing Cu toxicity to *M. mogurnda* and U toxicity to *H.viridissima*. However, the competition mechanism is not amenable where Cu toxicity to *H. viridissima* or U toxicity to *M. mogurnda* was not reduced, suggesting there is another mechanism in place. Perhaps further research could provide evidence, which would distinguish between physiological and toxicological effects. This could be achieved using metal tracers to compare the internal uptake and distribution of the metals by the organism, to the external metal concentration and bioavailability.

Comparing the individual protective effects of Ca and Mg could further define the effect of total hardness on metal toxicity. Although the present study did not investigate this subject, those studies that have (Carroll, 1979; Part *et al*, 1985; Jeffree and Simpson, 1986; Jackson *et al.*, 2000) reported Ca to be more effective than Mg in reducing the uptake and toxicity of trace metals to aquatic organisms. For example, Carroll *et al.* (1979) reported 7-fold more Mg was needed to ameliorate the toxicity of Cd to brook trout, *Salvelinus fontinalis*, to the same extent as Ca. Likewise, Jackson *et al.* (2000) reported increased Ca (ie. from 2.0 to 150 mg L^{-1}) decreased Cd toxicity to *Hyalella azteca* 14-fold (ie. LC_{50} increased from 3.8 to 55 µg L^{-1}), while increased Mg (ie. from 1.2 to 83 mg L^{-1}) reduced Cd toxicity 3-fold (ie. LC_{50} increased from 3.8 to 12 µg L^{-1}). The individual effects of Ca and Mg were not

investigated in this study. Further experimental work is required to define the individual protective effects of Ca and Mg to tropical Australian freshwater biota.

5.3 Effect of alkalinity on Cu and U toxicity

An increase in water hardness is frequently associated with an increase in alkalinity (ie. as Ca and/or Mg carbonate). For acidic waters (pH < 6), hardness and alkalinity are typically uncoupled, whereas in neutral and alkaline waters (pH 6–9) both parameters may be closely coupled. In this study, a 25-fold increase in alkalinity (from 4.0 to 102 mg L^{-1} as $CaCO_3$) at a fixed water hardness (165 mg L^{-1} as $CaCO_3$) and pH (6.0) did not significantly ($P > 0.05$) affect the toxicity of Cu or U to *H. viridissima*. In contrast, Markich *et al.* (1996) found that a 5-fold increase in alkalinity, at a fixed hardness (3.5 mg L^{-1} as $CaCO_3$) and pH (5.0), decreased U toxicity by 20%. The difference between studies may be attributed to either/or the use of different test organisms and diluent physico-chemical constituents. The effect of alkalinity on Cu and U toxicity to *M. mogurnda* was not assessed as the biological buffer (ie. MES), introduced to stabilise pH, enhanced sac-fry survival at elevated levels of Cu. Further work is needed to determine the effects of Cu and U toxicity on other freshwater organisms at varying alkalinity levels across a range of pH.

A 25-fold increase in alkalinity at constant hardness and pH, did not alter the toxicity of Cu or U to *H. viridissima*, implicating hardness (ie. Ca and Mg concentration) as the influential factor on Cu and U toxicity, under the experimental conditions described. The toxicity effect of Cu occurred without any change in speciation. However, increasing alkalinity altered U speciation through inorganic complexation, despite no change in U toxicity. These results are in agreement with those reported by Playle *et al.* (1992) who investigated Cu accumulation by fathead minnow (*Pimephales promelas*) gills. These authors found increasing Ca^{2+} reduced gill Cu accumulation, while increased carbonate did not. Similarly, Bradley and Sprague (1985) reported a 12-fold increase in hardness to reduce Zn toxicity to rainbow trout (*Salmo gairdneri*; renamed *Oncorhychus mykiss*) by more than one order of magnitude, while a two-fold increase in alkalinity had no effect. On the contrary, Daly *et al.* (1990b) found increasing alkalinity reduced Cu toxicity to an Australian freshwater shrimp, as did Markich *et al.* (1996) for U toxicity to a freshwater bivalve. These authors attributed the reduction of the free cupric ion activity to the formation of metal-carbonate complexes, which subsequently decreases the uptake and toxicity of the metal.

5.4 Derivation of water quality guidelines

Currently, there is debate as to which statistical endpoints should be used to derive water quality guidelines and assess environmental risk (Hoekstra and van Ewijk, 1993a,b; Denton and Norberg-King, 1996; Dhaliwal *et al.*, 1997; Moore and Caux, 1997). Much of this has been discussed by Markich and Camilleri (1997) and Camilleri *et al.* (1998) with respect to tropical ecotoxicology and is summerised below. The concentration of a toxicant, which has no adverse biological effect, has traditionally been reported as a NOEC value. This endpoint has come under criticism because it is restricted to one of the test concentrations, suggesting it does not necessarily represent the actual toxicant concentration that causes no adverse biological effect (Hoekstra and van Ewijk, 1993a,b; Chapman *et al.*, 1996; Moore and Caux, 1997). The determination of the NOEC is also reliant on the statistical power of the test, that is, the probability (*P*) to correctly conclude that the control is significantly different from the treatment concentration. Ecotoxicological tests often possess low power (ie. sometimes < 30%), thus it is difficult to accept the NOEC as a true measure of no biological effect (Hoekstra and van Ewijk, 1993a,b; Chapman *et al.*, 1996). For these reasons, Hoekstra and van Ewijk (1993a,b) have proposed the use of BEC_{10} as an alternative statistical measure to NOEC. Where BEC_{10} is the highest concentration which can be claimed with 95% confidence that its biological effect does not exceed 10% of the observed effect (Hoekstra and van Ewijk, 1993a). The process of deriving the BEC_{10} described by Hoekstra and van Ewijk (1993a), is summerised in Section 2.5.

In this study, most estimates of the BEC_{10} were lower than the corresponding NOEC values, with the exception of Cu toxicity values for *H.viridissima* at 165 mg L^{-1} hardness (Table 5.2). However, the difference between BEC_{10} and NOEC values does not seem dependent on metal, test organism, hardness or alkalinity. For example, the BEC_{10} value for the effect of 102 mg L^{-1} alkalinity on U toxicity to *H.viridissima* is 5-fold less than the NOEC, while the BEC_{10} for the effect of both 6.6 and 165 mg L^{-1} hardness is 2-fold less than the NOEC (Table 5.2). In contrast, Camilleri *et al.* (1998), reported the difference between BEC_{10} and NOEC values to be a product of inadequate test power, reflected by the inherent variability of organism response to the herbicide, Tebuthiuron. When species exhibiting less variability were tested (ie. *H. viridissima* and *M. macleayi* than *M. mogurnda*), replication increased, and more treatments tested, the NOEC better approximated the BEC_{10} (Camilleri *et al.*, 1998). This trend enabled the same authors to compare the predictive ability of BEC_{10} and NOEC values and concluded that the BEC_{10} should be considered an appropriate statistical endpoint to evaluate a no adverse biological concentration. However, Camilleri *et al.* (1998) warned that care should be taken that the BEC_{10} value does not result in an overly conservative estimate of the no adverse biological effect concentration.

The use of the LOEC as a measure of the lowest adverse biological effect concentration of a toxicant has come under the same criticism as the NOEC (Chapman *et al.*, 1996; Moore and Caux, 1997). Ahsanullah and Williams (1991) propose the minimum detectable effect concentration (MDEC) as an alternative statistical endpoint to the LOEC. The MDEC is calculated from a regression model and is defined as the metal concentration at which the response becomes significantly lower than in the 'control' treatment. The present study reported all estimates of the MDEC to be lower than the corresponding LOEC and EC_{50}/LC_{50} (Table 5.2). However, the LOEC estimates were sometimes close to or greater than the corresponding EC_{50}/LC_{50} values. The difference between LOEC and EC_{50}/LC_{50} values were generally less for *M. mogurnda* than those for *H. viridissima*. The small difference between the LOEC and LC_{50} values for *M. mogurnda* is most likely due to the large inherent variability of response (ie. survival) at each test concentration. However, this explanation cannot be applied where the LOEC equaled the EC_{50} calculated for U toxicity to *H. viridissima* at 102 mg L^{-1} alkalinity (Table 5.2). It is difficult to confidently use such LOEC values as an estimate of the true lowest adverse biological effect concentration.

The 1999 Australian water quality guideline for total Cu is 1.4 µg L^{-1} (depending on water hardness) to protect 95% of freshwater species (ANZECC, 1999). In this study, *H. viridissima* detected Cu at 0.8 µg L^{-1} and had an EC_{50} of 4.6 µg L^{-1}, at 6.6 mg L^{-1} hardness (Table 5.3). These values were not affected by increasing hardness and are within the proposed 1999 guidelines (Table 5.3). *Mogurnda mogurnda* detected Cu at 6.4 and 5.7 µg L^{-1}, at 6.6 and 330 mg L^{-1} hardness, respectively (Table 5.3). These BEC_{10} values are slightly greater than that proposed by the 1999 guidelines (Table 5.3). At 165 mg L^{-1} hardness, *M. mogurnda* detected Cu at 9.3 µg L^{-1}, two times greater than the proposed Cu guideline. Considering these values were derived in a synthetic water, which lacks any organic chelating agents (ie. DOC) and represents a high risk scenario, *H. viridissima* and *M. mogurnda* showed no adverse response to Cu at concentrations outside those listed in the 1999 guidelines.

A draft Australian guideline for U for the protection of freshwater ecosystems is 39 µg L^{-1} (R.A. van Dam, pers.comm.). Unlike several other metals (Cd, Cr(III), Cu, Ni, Pb and Zn), there is currently no provision in the guidelines to use an algorithm to modify the U guideline value to account for increased water hardness. The present study found *H. viridissima* detected U at 14 µg L^{-1} in water with a hardness of 6.6 mg L^{-1} $CaCO_3$ (Table 5.3), which is below the draft guideline. However, *H. viridissima* detected U at 81 and 47 µg L^{-1} in water with a hardness of 165 and 330 mg L^{-1} $CaCO_3$, respectively (Table 5.3). These BEC_{10} values are approximately two times greater than the draft U guideline. *Mogurnda mogurnda* detected U at levels about 24 times the draft U guideline (ie. second

Table 5.3: Toxicity endpoints (BEC_{10}, MDEC, NOEC, LOEC, EC_{50}, LC_{50}) calculated for *H. viridissima* and *M. mogurnda* exposed to Cu and U (µg L^{-1}) at three hardness levels and two alkalinity levels (pH 6.0 ± 0.3) for 96 h.

Species	Metal	Hardness (mg L^{-1} as $CaCO_3$)	Alkalinity (mg L^{-1} as $CaCO_3$)	BEC_{10}	MDEC	NOEC	LOEC	Effect Concentration (95% C.I)
Green hydra *(H. viridissima)*	Cu	6.6	4	0.8	1	0.9	1.9	4.6[a] (4.1-5.1)
		165	4	1.1	1.4	0.9	1.7	5.0[a] (4.5-5.5)
		165	102	1.2	1.4	0.7	1.8	6.0[a] (5.5-6.5)
		330	4	0.8	0.9	0.9	1.8	5.5[a] (5.0-6.0)
	U	6.6	4	14	32	32	62	114[a] (107-121)
		165	4	81	90	150	162	177[a] (166-188)
		165	102	25	42	130	171	171[a] (150-192)
		330	4	47	62	62	87	219[a] (192-246)
Purple-spotted gudgeon *(M. mogurnda)*	Cu	6.6	4	6.4	8.8	11.4	11.8	13.0[b] (10.8-15.2)
		165	4	9.3	10.6	20	23.1	26.4[b] (23.1-29.7)
		330	4	5.7	6.9	19.7	24.4	23.4[b] (16.1-30.7)
	First investigation							
	U	6.6	4	1410	1460	1450	1530	1730[b] (1600-1860)
		165	4	570	860	1100	1310	1335[b] (1165-1500)
		330	4	725	915	1050	1280	1270[b] (1140-1400)
	Second investigation							
	U	6.6	4	900	1220	1835	1950	1965[b] (1600-2325)
		165	4	1110	1240	1510	1770	1710[b] (1400-2000)
		330	4	860	1040	1530	1990	1770[b] (1570-1970)

[a] 50% Effect concentration (EC_{50}).

[b] 50% Lethal concentration (LC_{50}).

investigation; Table 5.3). Unlike the sensitivity of *H. viridissima* to U, increasing hardness did not affect the sensitivity of *M. mogurnda* (Table 5.3). Although the draft U guideline appears conservative with respect to *M. mogurnda* toxicity data, the fact that *M. mogurnda* is eight-fold less sensitive to U than *H. viridissima* must be considered.

5.5 Conclusions

The influence of true water hardness and alkalinity on Cu and U toxicity to tropical Australian freshwater species was investigated, to help modify national water quality guidelines, and because such data is limited and/or ambiguous for tropical ecosystems.

The present study provides evidence that the toxicity of U to *H. viridissima* is reduced with increasing hardness, while U toxicity to *M. mogurnda* is not affected. In contrast, increasing hardness reduced the toxicity of Cu to *M. mogurnda*, while it had no affect on the toxicity of Cu to *H. viridissima*. Further work is needed to determine the effects of Cu and U on other freshwater organisms at varying hardness levels, to determine if a generic relationship exists which will allow an algorithm to be established that can be used to modify the national guideline on a site-specific basis. Markich and Jeffree (1994) proposed that Ca concentration is a better choice than total hardness (Ca + Mg) for the protection of freshwater biota because Ca is far more effective at ameliorating metal toxicity at the cell membrane surface than Mg. They suggest that only in surface waters where the concentration of Mg considerably exceeds that of Ca will the joint hardness (Ca + Mg) be more useful. The German water quality guidelines actually use Ca concentration instead of total hardness with Cu, Zn and Cd for the protection of freshwater fisheries (Rump and Krist 1992). This study also found alkalinity had no effect on Cu and U toxicity to *H.viridissima*, suggesting that true water hardness is more important than alkalinity in reducing metal toxicity. Copper speciation did not differ with increasing hardness or alkalinity, eliminating it is a confounding factor. In contrast, U speciation was altered through inorganic complexation. It is speculated that hardness (ie. Ca^{2+} and Mg^{2+}) reduced Cu and U toxicity by reducing the uptake of the free metal ion at the cell membrane surface. A mechanistic knowledge of metal toxicity is important for improving quality guidelines for the protection of freshwater biota on a site-specific basis.

In summary, the information reported in this study will assist the development of water quality guidelines to protect tropical Australian freshwater systems. The separation of hardness and alkalinity effects provided a mechanistic knowledge of the influence these parameters have on Cu and U toxicity in tropical freshwaters. Such information is important for improving national water quality guidelines and for the protection of freshwater biota on a site-specific basis.

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Appendix A Summary of Cu and U toxicity data for Australian freshwater biota[a,o]

Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})	Reference
Chlorophyta									
Alga *(Chlorella sp.)*	Copper	Synthetic Magela Creek	5.7	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	72 h growth inhibition	1.1 (BEC_{10}) 1.4 (MDEC) 35 (EC_{50}) (28-42)	Franklin *et al.* (1998)
	Copper	Synthetic Magela Creek	6.5	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	72 h growth inhibition	0.6 (BEC_{10}) 0.7 (MDEC) 1.5 (EC_{50}) (0.8-2.8)	Franklin *et al.* (1998)
	Uranium	Synthetic Magela Creek	5.7	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	72 h growth inhibition	21 (BEC_{10}) 34 (MDEC) 78 (EC_{50}) (71-83)	Franklin *et al.* (1998)
	Uranium	Synthetic Magela Creek	6.5	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	72 h growth inhibition	11 (BEC_{10}) 13 (MDEC) 44 (EC_{50}) (39-49)	Franklin *et al.* (1998)
Cnidaria									
Green hydra *(Hydra viridissima)*	Copper	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	96 h population growth	1.6 (BEC_{10})[c] 1.8 (MDEC)[d] 4.0 (EC_{50})[e] (3.8-4.2)	Markich & Camilleri (1997)
	Copper	Autoclaved carbon-filtered mains	7.2 ± 0.4	20	NR	NR	96 h population growth	4 (NOEC) 8 (LOEC) 8.5 (LC_{50})	Pollino & Holdway (1999)

Appendix A Cont'd

Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Green hydra *(Hydra viridissima)*	Copper	Synthetic Magela Creek	6.0 ± 0.1	6.6	4	< 0.2 (D)	96 h population growth	0.8 (BEC_{10}) 1.0 (MDEC) 0.9 (NOEC) 1.9 (LOEC) 5.1 (EC_{50}) (3.3-6.9)	Riethmuller *et al.* (This study)
	Copper	Synthetic Magela Creek	6.0 ± 0.1	165	4	< 0.2 (D)	96 h population growth	1.1 (BEC_{10}) 1.4 (MDEC) 0.9 (NOEC) 1.7 (LOEC) 5.1 (EC_{50}) (4.2-5.9)	Riethmuller *et al.* (This study)
	Copper	Synthetic Magela Creek	6.0 ± 0.1	165	102	< 0.2 (D)	96 h population growth	1.2 (BEC_{10}) 1.4 (MDEC) 0.7 (NOEC) 1.8 (LOEC) 6.0 (EC_{50}) (3.8-8.3)	Riethmuller *et al.* (This study)
	Copper	Synthetic Magela Creek	6.0 ± 0.1	330	4	< 0.2 (D)	96 h population growth	0.8 (BEC_{10}) 0.9 (MDEC) 0.9 (NOEC) 1.8 (LOEC) 6.1 (EC_{50}) (5.2-6.9)	Riethmuller *et al.* (This study)
	Uranium	Buffalo Billabong	6.5 ± 0.2	4[l] (3-5)	3[l] (2-4)	NR[g]	96 h population growth	160 (LOEC)[a] (Dry season) 194 (LOEC) (Wet season)	Allison & Holdway (1988)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	96 h population growth	56 (BEC_{10}) 61 (MDEC) 108 (EC_{50}) (102-114)	Markich & Camilleri (1997)

Appendix A Cont'd

Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Green hydra *(Hydra viridissima)*	Uranium	Synthetic Magela Creek	6.0 ± 0.1	6.6	4	< 0.2 (D)	96 h population growth	14 (BEC_{10}) 32 (MDEC) 32 (NOEC) 62 (LOEC) 123 (EC_{50}) (113-132)	Riethmuller *et al.* (This study)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	165	4	< 0.2 (D)	96 h population growth	81 (BEC_{10}) 90 (MDEC) 150 (NOEC) 162 (LOEC) 184 (EC_{50}) (175-193)	Riethmuller *et al.* (This study)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	165	102	< 0.2 (D)	96 h population growth	25 (BEC_{10}) 42 (MDEC) 130 (NOEC) 171 (LOEC) 186 (EC_{50}) (167-204)	Riethmuller *et al.* (This study)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	330	4	< 0.2 (D)	96 h population growth	47 (BEC_{10}) 62 (MDEC) 62 (NOEC) 87 (LOEC) 218 (EC_{50}) (173-262)	Riethmuller *et al.* (This study)
Pink hydra *(Hydra vulgaris)*	Copper	Autoclaved carbon-filtered mains	7.2 ± 0.4	20	NR	NR	96 h population growth	4 (NOEC) 8 (LOEC) 26 (LC_{50})	Pollino & Holdway (1999)
	Uranium	Buffalo Billabong	6.4 ± 0.1	4[f] (3-5)	3[f] (2-4)	NR	96 h population growth	740 (LOEC) (Dry season)	Allison & Holdway (1988)

Appendix A Cont'd

Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Mollusca									
Mussel (*Velesunio angasi*)	Copper	Tap	7.5 ± 0.1	54[i] (51-57)	27[i] (25-29)	NR	96 h survival	21000 (LC_{50})[j] (13000-32000)	Skidmore & Firth (1983)
							792 h (33 d) survival	420 (LC_{50})	Skidmore (1986)
							1320 h (55 d) survival	~ 210 (LC_{50})	
	Uranium	Synthetic Magela Creek	5.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	48 h behaviour	62 (BEC_{10})[k] 89 (EC_{50}) (83-95)	Markich (1998)
								92 (BEC_{10})[l] 117 (EC_{50}) (113-121)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	5.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	3.7 (D)	48 h behaviour	78 (BEC_{10})[k] 112 (LC_{50}) (101-123)	Markich (1998)
								113 (BEC_{10})[l] 144 (EC_{50}) (138-150)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	5.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	8.9 (D)	48 h behaviour	138 (BEC_{10})[k] 194 (LC_{50}) (188-200)	Markich (1998)
								197 (BEC_{10})[l] 247 (EC_{50}) (240-254)	Markich *et al.* (1996)

Appendix A Cont'd

Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Mussel (*Velesunio angasi*)	Uranium	Synthetic Magela Creek	5.3 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	48 h behaviour	73 (BEC_{10})[k] 106 (LC_{50}) (96-116)	Markich (1998)
								108 (BEC_{10})[l] 141 (EC_{50}) (135-147)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	5.5 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	48 h behaviour	85 (BEC_{10})[k] 126 (LC_{50}) (114-138)	Markich (1998)
								125 (BEC_{10})[l] 163 (EC_{50}) (156-170)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	5.5 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	3.7 (D)	48 h behaviour	133 (BEC_{10})[k] 190 (LC_{50}) (184-196)	Markich (1998)
								192 (BEC_{10})[l] 242 (EC_{50}) (233-251)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	5.5 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	8.9 (D)	48 h behaviour	291 (BEC_{10})[k] 399 (LC_{50}) (381-417)	Markich (1998)
								399 (BEC_{10})[l] 497 (EC_{50}) (477-517)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	5.8 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	48 h behaviour	130 (BEC_{10})[k] 210 (LC_{50}) (191-229)	Markich (1998)
								214 (BEC_{10})[l] 290 (EC_{50}) (275-303)	Markich *et al.* (1996)

Appendix A Cont'd

Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[a]	Reference
Mussel *(Velesunio angasi)*	Uranium	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	48 h behaviour	244 (BEC_{10})[k] 446 (LC_{50}) (427-465)	Markich (1998)
								416 (BEC_{10})[l] 634 (EC_{50}) (606-662)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	3.7 (D)	48 h behaviour	362 (BEC_{10})[k] 597 (LC_{50}) (559-635)	Markich (1998)
								558 (BEC_{10})[l] 824 (EC_{50}) (786-862)	Markich *et al.* (1996)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	8.9 (D)	48 h behaviour	635 (BEC_{10})[k] 941 (LC_{50}) (888-994)	Markich (1998)
								913 (BEC_{10})[l] 1228 (EC_{50}) (1188-1268)	Markich *et al.* (1996)
Crustacea									
Shrimp *(Caridina sp.)*	Copper	Synthetic Gulungul Creek	6.0 ± 0.1	27 (25-30)	5	< 0.5 (T)	48 h survival	4.5 (LC_{50}) (2-8)	Williams *et al.* (1991)
							72 h survival	4 (LC_{50}) (2-6)	
							96 h survival	3.5 (LC_{50}) (2-5)	
Water Flea *(Daday a macrops)*	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	24 h survival	1254 (LC50) (923-1660)	Bywater *et al.* (1991)

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Water Flea *(Diaphanosoma excisum)*	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	24 h survival	1140 (LC50) (787-1570)	Bywater *et al.* (1991)
Water Flea *(Latonopsis fasciculata)*	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	24 h survival	467 (LC50) (365-593)	Bywater *et al.* (1991)
Prawn *(Macrobrachium sp.)*	Copper	Magela Creek	7.0 ± 0.1	10	NR	NR	96 h survival	170 (LC_{50})	Giles (1974)
	Copper	Tap	7.5 ± 0.1	54 (51-57)	27 (25-29)	NR	48 h survival	170 (LC_{50})	Skidmore & Firth (1983)
							96 h survival	160 (LC_{50})	
	Uranium	Magela Creek	7.0 ± 0.1	10	NR	NR	96 h survival	> 5700 (LC_{50})	Giles (1974)
Water Flea *(Moinodaphnia maceayli)*	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3-6)	NR	24 h survival	1470 (LC50) (1210-1700)	Bywater *et al.* (1991)
	Uranium	Magela Creek	6.5 ± 0.1	4[f] (3-5)	3[f] (2-4)	NR	48 h survival	211 (LC50) (200-222)	eriss
	Uranium	Magela Creek	6.5 ± 0.1	4[f] (3-5)	3[f] (2-4)	NR	120 h reproduction	20 (NOEC)[m] 22 (LOEC) 44 (EC50) (41-47)	eriss
Chordata									
Chanda Perch *(Ambassis castelnaui)*	Copper	Tap	7.5 ± 0.1	54 (51-57)	27 (25-29)	NR	48 h survival	200 (LC_{50})	Skidmore (1986)
							96 h survival	140 (LC_{50})	

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Reticulated Perch (*Ambassis macleayi*)	Uranium	Magela Creek	6.6 ± 0.1	16 (15-17)	4.8 (4.6-5.0)	NR	48 h survival	910 (LC_{50}) (627-1230)	Bywater *et al.* (1991)
							72 h survival	910 (LC_{50}) (627-1230)	
							96 h survival	910 (LC_{50}) (627-1230)	
Striped Grunter (*Amniataba percoides*)	Uranium	Magela Creek	7.0 ± 0.1	10	NR	NR	96h survival	2850 (LC_{50})	Giles (1974)
Mariana's Hardyhead (*Craterocephalus marianae*)	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	2120 (LC50) (1640-2500)	Bywater *et al.* (1991)
							72 h survival	1390 (LC50) (935-1840)	
							96 h survival	1390 (LC50) (935-1840)	
Marjorie's Hardyhead (*Craterocephalus marjoriae*)	Copper	Magela Creek	7.0 ± 0.1	10	NR	NR	96 h survival	40 (LC_{50})	Giles (1974)
	Uranium	Magela Creek	7.0 ± 0.1	10	NR	NR	96 h survival	4850 (LC_{50})	Giles (1974)
Fly-specked Hardyhead (*Craterocephalus stercusmuscarum*)	Copper	Buffalo Billabong	6.9 ± 0.1	6.6	NR	NR	96 h survival	17 (LC_{50}) (16-27)	Baker & Waldon (1984)

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Penny Fish (*Denariusa bandata*)	Copper	Synthetic Gulungul Creek	6.0 ± 0.1	27 (25-30)	5	< 0.5 (T)	48 h survival	140 (LC_{50}) (105-195)	Williams *et al.* (1991)
							72 h survival	120 (LC_{50}) (88-170)	
							96 h survival	77 (LC_{50}) (42-120)	
Carp Gudgeon (*Hypseleostris compressus*)	Copper	Ja Ja Billabong	6.0 ± 0.4	8[n] (6-10)	4[n] (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	330 (LC_{50})	R Bolus & J Skidmore (Pers comm)
	Uranium	Ja Ja Billabong	6.0 ± 0.4	8[n] (6-10)	4[n] (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	7520 (LC_{50})	R Bolus & J Skidmore (Pers comm)
Spangled Grunter (*Madigania unicolor*)	Uranium	Magela Creek	7.0 ± 0.1	10	NR	NR	96 h survival	4670 (LC_{50})	Giles (1974)
Black-Striped Rainbowfish (*Melanotaenia nigrans*)	Copper	Ja Ja Billabong	6.0 ± 0.4	8[n] (6-10)	4[n] (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	230 (LC_{50})	R Bolus & J Skidmore (Pers comm)
	Copper	Buffalo Billabong	7.2 ± 0.2	25	18	NR	96 h survival	130 (LC_{50}) (100-140)	Baker & Waldon (1984)
	Uranium	Ja Ja Billabong	6.0 ± 0.4	8[n] (6-10)	4[n] (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	5130 (LC_{50})	R Bolus & J Skidmore (Pers comm)
	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	2400 (LC_{50}) (1900-2780)	Bywater *et al.* (1991)
							72 h survival	2140 (LC_{50}) (1425-2930)	
							96 h survival	1940 (LC_{50}) (1410-1590)	

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Black-Striped Rainbowfish (*Melanotaenia nigrans*)	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	2700 (LC_{50}) (1870-3510)	Bywater *et al.* (1991)
							72 h survival	2250 (LC_{50}) (1810-2670)	
							96 h survival	2160 (LC_{50}) (1740-2600)	
Chequered Rainbowfish (*Melanotaenia splendida inornata*)	Copper	Ja Ja Billabong	6.0 ± 0.4	8[n] (6-10)	4[n] (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	750 (LC_{50})	R Bolus & J Skidmore (Pers comm)
	Copper	Magela Creek	6.9 ± 0.1	3.3	6.7	NR	96 h survival	60 (LC_{50}) (40-90)	Baker & Waldon (1984)
	Copper	Tap	7.5 ± 0.1	54 (51-57)	27 (25-29)	NR	96 h survival	460 (LC_{50})	Skidmore (1986)
							192 h survival	340 (LC_{50})	
							720 h (30 d) reproduction	12 (NOEC) 18 (LOEC)	
	Copper	Synthetic Gulungul Creek	6.0 ± 0.1	27 (25-30)	5	NR	48 h survival	210 (LC50) (175-250)	Williams *et al.* (1991)
							72 h survival	205 (LC50) (175-240)	
							96 h survival	168 (LC50) (140-195)	
	Uranium	Ja Ja Billabong	6.0 ± 0.4	8 (6-10)	4 (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	6840 (LC_{50})	R Bolus & J Skidmore (Pers comm)

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Chequered Rainbowfish (*Melanotaenia splendida inornata*)	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	3140 (LC_{50}) (2590-3830)	Bywater *et al.* (1991)
							72 h survival	3030 (LC_{50}) (2470-3740)	
							92 h survival	3030 (LC_{50}) (2470-3740)	
	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	4380 (LC_{50}) (2975-8730)	Bywater *et al.* (1991)
							72 h survival	3940 (LC_{50}) (2680-7490)	
							92 h survival	3944 (LC_{50}) (2680-7490)	
	Uranium	Buffalo Billabong	6.6 ± 0.2	5.1	3.2	5.8 (D)	96 h survival	1585 (LC_{50}) (1250-2000)	Holdway (1992)
	Uranium	Buffalo Billabong	6.3 ± 0.2	4.1 (4.0-4.2)	1.8 (1.7-1.9)	1.5 (< 0.1-4) (D) 2.7 (0.8-4.6) (T)	168 h (7 d) survival	1790 (LC_{50}) (1540-2420)	Holdway (1992)
	Copper	Tap	7.5 ± 0.1	54 (51-57)	27 (25-29)	NR	96 h survival	200 (LC_{50})	Skidmore & Firth (1984)
Purple-spotted Gudgeon (*Mogurnda mogurnda*)	Copper	Buffalo Billabong	6.5	4[f] (3-5)	3[f] (2-4)	NR	96 h sac-fry survival	20 (NOEC) 64 (LOEC)	Rippon & Hyne (1992)
							120 h embyro hatching	> 200 (LOEC)	
	Copper	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	96 h survival	12 (BEC_{10}) 13 (MDEC) 23 (LC_{50}) (22-24)	Markich & Camilleri (1997)

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[i]	Reference
Purple-spotted Gudgeon (*Mogurnda mogurnda*)	Copper	Synthetic Magela Creek	6.0 ± 0.1	6.6	4	< 0.2 (D)	96 h survival	6.4 (BEC_{10}) 8.8 (MDEC) 11.4 (NOEC) 11.8 (LOEC) 12.9 (EC_{50}) (9.3-16.6)	Riethmuller *et al.* (This study)
	Copper	Synthetic Magela Creek	6.0 ± 0.1	165	4	< 0.2 (D)	96 h survival	9.3 (BEC_{10}) 10.6 (MDEC) 20 (NOEC) 23.1 (LOEC) 25.9 (EC_{50}) (21.0-30.7)	Riethmuller *et al.* (This study)
	Copper	Synthetic Magela Creek	6.0 ± 0.1	330	4	< 0.2 (D)	96 h survival	5.7 (BEC_{10}) 6.9 (MDEC) 19.7 (NOEC) 24.4 (LOEC) 24.6 (EC_{50}) (16.6-32.6)	Riethmuller *et al.* (This study)
	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	2340 (LC_{50}) (1860-2790)	Bywater *et al.* (1991)
							72 h survival	1265 (LC_{50}) (950-1650)	
							92 h survival	1265 (LC_{50}) (950-1650)	
	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	2450 (LC_{50}) (1960-2990)	Bywater *et al.* (1991)
							72 h survival	1665 (LC_{50}) (1280-2170)	
							92 h survival	1665 (LC_{50}) (1280-2170)	

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[i]	Reference
Purple-spotted Gudgeon (*Mogurnda mogurnda*)	Uranium	Buffalo Billabong	6.4 ± 0.1	3.2 (3.0-3.4)	3 (2.8-3.2)	5.1 (4.5-5.7) (D) 5.4 (4.8-6.0) (T)	336 h (14 d) survival	1000 (NOEC) 2040 (LOEC)	Holdway (1992)
							336 h (+ 360 h post exposure)	502 (NOEC) 1000 (LOEC)	
	Uranium	Buffalo Billabong	6.3 ± 0.2	4.1 (4.0-4.2)	1.8 (1.7-1.9)	1.5 (< 0.1-4) (D) 2.7 (0.8-4.6) (T)	168 h (7 d) survival	1810 (LC_{50}) (1730-1780)	Holdway (1992)
							168 h (+ 168 h post exposure)	1015 (LC_{50}) (900-1190)	
							168 h (7 d) growth	920 (NOEC) 1780 (LOEC)	
							168 h (+ 168 h post exposure)	< 455 (NOEC) 455 (LOEC)	
	Uranium	Buffalo Billabong	6.6 ± 0.2	5.1	3.2	5.8 (D)	96 h survival	1790 (LC50) (1385-2100)	Holdway (1992)
							96 h growth	640 (NOEC) 1240 (LOEC)	
	Uranium	Buffalo Billabong	6.3 ± 0.2	5.1	3.2	5.8 (D)	96 h survival	3750 (LC_{50}) (2580-4925)	Holdway (1992)
							168 h (7 d) survival	3070 (LC_{50}) (2580-3590)	
							168 (+168 h post exposure)	1640 (LC_{50}) (1120-2565)	
							168 h growth	2580 (NOEC) 4930 (LOEC)	
							168 h (+ 168 h post exposure)	1240 (NOEC) 2580 (LOEC)	

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[i]	Reference
Purple-spotted Gudgeon *(Mogurnda mogurnda)*	Uranium	Buffalo Billabong	6.6 ± 0.2	5.1	3.2	5.8 (D)	96 h survival	3750 (LC_{50}) (2580-4925)	Holdway (1992)
							168 h (7 d) survival	3750 (LC_{50}) (2580-4925)	
							168 (+168 h post exposure)	3078 (LC_{50}) (2580-3590)	
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	3.9 (3.8-4.0)	4.1 (4.0-4.2)	< 0.2 (D)	96 h survival	1270 (BEC_{10}) 1300 (MDEC) 1570 (LC_{50}) (1510-1630)	Markich & Camilleri (1997)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	6.6	4	< 0.2 (D)	96 h survival	900 (BEC_{10}) 1220 (MDEC) 1835 (NOEC) 1947 (LOEC) 2002 (EC_{50}) (1870-2133)	Riethmuller *et al.* (This study)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	165	4	< 0.2 (D)	96 h survival	1110 (BEC_{10}) 1240 (MDEC) 1511 (NOEC) 1773 (LOEC) 1713 (EC_{50}) (1519-1908)	Riethmuller *et al.* (This study)
	Uranium	Synthetic Magela Creek	6.0 ± 0.1	330	4	< 0.2 (D)	96 h survival	860 (BEC_{10}) 1040 (MDEC) 1533 (NOEC) 1989 (LOEC) 1876 (EC_{50}) (1713-2038)	Riethmuller *et al.* (This study)

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Species	Metal	Water type	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	Organic carbon[b] (mg L^{-1})	Test Endpoint	Water Concentration (µg L^{-1})[j]	Reference
Eel-tailed Catfish *(Porochilus rendahli)*	Copper	Synthetic Gulungul Creek	6.0 ± 0.1	25-30	5	< 0.5 (T)	48 h survival	210 (LC_{50}) (160-250)	Williams *et al.* (1991)
							72 h survival	85 (LC_{50}) (17-125)	
Blue Eye *(Pseudomugil tenellus)*	Copper	Ja Ja Billabong	6.0 ± 0.4	8[n] (6-10)	4[n] (2-6)	11 (7.3-14.7) (D) 14 (9.5-18.5) (T)	96 h survival	120 (LC_{50})	R Bolus & J Skidmore (Pers comm)
	Uranium	Magela Creek	6.6 ± 0.1	4.8 (4.6-5.0)	3.3 (3.0-3.6)	NR	48 h survival	940 (LC_{50}) (640-1230)	Bywater *et al.* (1991)
							72 h survival	830 (LC_{50}) (570-1070)	
							96 h survival	830 (LC_{50}) (570-1070)	

Appendix A Cont'd

[a] All numerical values represent mean values, or their range, with 95% confidence intervals (C.I) in parentheses (where reported). Means shown with ± values were regulated within the reported limits. NR: not reported. Uranium (U) concentration is expressed as uranyl (ie. UO_2); this was derived by multiplying the U concentration by 1.14.

[b] T, total; D, dissolved.

[c] BEC_{10}, 10% bounded effect concentration (Hoekstra and van Ewijk, 1993), an analogous statistical measure of the no-observed effect concentration (NOEC).

[d] MDEC, minimal detectable concentration (Ahsanullah and Williams, 1991), an analogous statistical measure of the lowest-observed effect concentration (LOEC).

[e] EC_{50}, median effect concentration.

[f] Estimated from established protocols (ie. Holdway and Wiecek, 1988; Allison *et al.*, 1991; Holdway, 1992; Hyne *et al.*, 1995).

[g] Not reported.

[h] LOEC, lowest-observed effect concentration.

[i] Estimated from the mean physico-chemistry of Sydney tap water.

[j] LC_{50}, concentration at which there is 50% survival.

[k] The frequency of valve adductions (ie. movements) was the measured behavioural characteristic.

[l] The duration of valve gape was the measured behavioural characteristic.

[m] NOEC, no-observed effect concentration.

[n] Estimated from the mean physico-chemistry of Ja Ja Billabong during the Dry season of 1982 (NTDTW, 1983).

[o] Modified from Markich and Camilleri (1997).

Appendix B Test protocols

B.1 Green hydra (*Hydra viridissima*) population growth test

B.1.1 Objective

The objective of the test series (ie. 3-4 definative tests) was to determine the concentration of a specified chemical that shows:

a) A no-observed effect concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This is compared to the 10% bounded effect concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra and Van Ewijk, 1993);

b) The lowest-observed effect concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This is compared to the minimum detectable effect concentration (MDEC), which is defined as the metal concentration at which the response became significantly ($P \leq 0.05$) lower than that of control (Ahsanullah and Williams, 1991), and

c) The median effect concentration, where a 50% decline is found. This is measured as the EC_{50} on the population growth of *H. viridissima* (Green hydra) over 96 h.

B.1.2 Principle of the test

Asexually reproducing (budding) test hydra are exposed to a range of chemical concentrations for 96 h. Observations of any changes to the hydra population (ie. changes in the number of intact hydroids, where one hydroid equals one animal plus any attached buds) are recorded at 24 h intervals. The method is based on the 'Hydra population growth test' described by Hyne *et al.* (1996).

B.1.3 Test organism

The species is *Hydra viridissima* (Cnidaria, Hydrozoa). *H. viridissima* is referred to as 'green' hydra because of its green colouration resulting from the presence of a symbiotic green alga in the gastrodermal cells of the animal. Although the precise distribution of this species has not been mapped, it has been found in a variety of aquatic habitats in northern Australia. Test hydra were obtained from laboratory cultures as described in Appendix C.1.1. Test organisms are selected on the basis that the hydroid is bearing one tentacled bud. Asexual budding is a characteristic of hydra in optimal environmental conditions. Hydra selected for testing must be free of overt disease and gross morphological deformity (ie. show no signs of clubbing or contraction).

B.1.4 Synthetic water

The test water is an artificial or 'synthetic' water that simulates the inorganic composition of Magela Creek water during the Wet season. Magela Creek water is very soft, slightly acidic and has a low buffering and complexation capacity. These qualities are predicted to maximise the toxic response of an organism, and hence, provide the greatest probability of detriment to organisms exposed to metals. The ionic composition of Magela Creek water is representative of sandy braided streams throughout much of the Wet/Dry tropics. The synthetic water is prepared by adding analytical grade reagents to deionised (DI) water (< 1µS cm^{-1}) in acid-washed polyethylene containers, as close as practical to the start of the test. The pH of the test water is adjusted to the required level (in this case 6.0 ± 0.15) with dilute acid and/or base. The test water should be stored in sealed polyethylene containers and refrigerated (4°C) until use.

Mean Nominal Composition of the Synthetic Water

Physico-chemical Parameter	Background Water
pH	6.0 ± 0.15[a]
Temperature (°C)	27 ± 1[a]
Na (mg L^{-1})	1.00
K (mg L^{-1})	0.37
Ca (mg L^{-1})	0.45
Mg (mg L^{-1})	0.60
Cl (mg L^{-1})	2.32
SO_4 (mg L^{-1})	3.12
HCO_3 (mg L^{-1})	2.63
NO_3 (µg L^{-1})	0.07
Fe (µg L^{-1})	100
Al (µg L^{-1})	70
Mn (µg L^{-1})	9.7
U (µg L^{-1})	0.10
Cu (µg L^{-1})	0.70
Zn (µg L^{-1})	0.70
Pb (µg L^{-1})	0.12

B.1.5 Stock solutions

Analytical grade reagents are used to prepare stock solutions. A stock solution of the appropriate chemical is prepared in a clean, inert container and refrigerated (4°C). The source of the stock solution (eg. date of preparation, by whom), is described on an information sheet.

B.1.6 Test solutions

Test solutions are prepared by serially diluting a stock solution with pH-adjusted synthetic water. The pH is then re-adjusted if necessary, using 0.02 M HNO_3 or 0.0125 M NaOH. Test solution concentrations are determined from the results of range-finding studies. Test solutions are prepared in bulk at the start of a test in 5 L polyethylene screw-topped containers and refrigerated (4°C) until required. Alternatively, test solutions are prepared daily if it is established that the toxicity of the test solution varies significantly when stored for the test period.

B.1.7 Apparatus and test equipment

All materials that come into contact with any liquid into which the hydra are placed, or the hydra themselves, should be chemically inert.

Container preparation

All containers (ie. vials, bottles, Petri dishes and lids etc) and Pasteur pipettes used in any part of the test are prepared in the following manner:

- Undergo a dish washer (Gallay Laboratory 999) cycle, containing detergent (Gallay Clean A phosphate free) and acid (double strength), using Milli-RO water for two rinse cycles;
- Rinse with Milli-Q water (< 1 µS cm^{-1}); and
- Allow to air dry.

OR

- Immerse in a 1-3% detergent solution (eg. Decon Neutracon) for up to 24 h;
- Scrub to remove extraneous material, then rinse thoroughly in tap water;
- Immediately immerse in a 5% HNO_3 solution for up to 24 h;
- Thoroughly rinse at least 3 times with Milli-Q water (< 1 µS cm^{-1}); and
- Allow to air dry.

Note: Immediately before use the containers should be rinsed with pH-adjusted synthetic water. Other equipment should be rinsed thoroughly with Milli-Q water (< 1 $\mu S\ cm^{-1}$) before use.

Temperature control

Tests were conducted at 27 ± 1°C using a constant temperature incubator. The temperature of the test containers are maintained at 27 ± 1°C by the use of warming trays set at 27°C on the microscope bench after they are removed from the incubator for observation.

Photoperiod control

Tests were conducted with a 12 h light: 12 h dark photoperiod, where the mid-point coincides with solar midday. Light intensity should be typical for normal laboratory working conditions (ie. 30-100 $\mu Em^{-2}s^{-1}$ Photosynthetic Active Radiation).

Equipment

- Eight 5 L polyethylene containers (to hold treatment solutions)
- Refrigerator for storage of test and stock solutions
- Twenty-four 90 mm diameter disposable plastic Petri dishes with lids
- Sixteen 150 mL plastic Nalgene® beakers (for water parameter measurement)
- Twenty-four 50 mL plastic Nalgene® beakers (for aliquoting 30 mL to respective treatment Petri dishes)
- Maximum-minimum thermometers
- Calibrated mercury thermometer
- pH meter, pH probe, and pH buffer solutions of 6.87 and 4.01
- Conductivity meter and probe
- Dissolved oxygen meter fitted with a micro-oxygen electrode
- Binocular dissecting microscope with bright field/dark field illumination
- Automatic 0-50 mL dispenser
- Three clear perspex trays, each capable of holding 8 Petri dishes, with position numbers 1 to 24 marked
- Laboratory warming trays, set at 27°C, capable of accommodating the clear plastic trays
- Random number generator

- Two plastic trays, one of such a size to hold sixteen 50 mL beakers and the other to hold twenty-four 50 mL beakers
- Pasteur pipettes, with internal tip diameter of ~ 2 mm

B.1.8 Test environment

The preparation and storage of test solutions, culturing of test hydra, and conducting tests should be carried out in premises free from harmful vapour, dust, and any undue disturbance. All workers involved in any part of the test should wash hands and arms thoroughly with fragrance-free soap and rinse well with tap water before commencing any part of the test procedure.

B.1.9 Data recording

Test animals are observed and data recorded at 24 h intervals after the commencement of the test (when t = 0 h). Observations made at the end of the first 24 h period are designated as Day 1 observations; at the end of the second 24 h period, Day 2 observations etc. Water parameters are measured and adjusted (where appropriate) and recorded at the beginning and end of each 24 h period, and are designated as Fresh Water Day 1, 24 h -old Water Day 1, respectively, and so forth during the test.

B.1.10 Test procedure

Day 1

1. Prepare the test solutions (as outlined in Section B.1.6) and leave at room temperature.
2. Isolate approximately 250 suitable hydra in synthetic water in a Petri dish and leave at room temperature. A 'suitable test hydra' is a hydra with one bud. The bud must not be fully developed (ie. tentacles are present only as 'bumps', and the bud must not appear ready to detach from the main stem of the hydroid).
3. Dispense 30 mL aliquots of each test concentration (normally 8) into 3 appropriately labelled replicate Petri dishes (ie. 3 x 30 mL for each test solution), and arrange in three replicate groups on clear plastic trays (eg. Control replicate 1 to X $\mu g\ L^{-1}$ on Tray 1).
4. Using a microscope and Pasteur pipette, pick out one hydra from the isolated stock and place into Control replicate 1.
5. Repeat for remaining test concentrations of replicate 1, working up in concentration, and ending with the highest concentration.
6. Discard the used pipette and select a new one.
7. Repeat steps 4-6 until all test dishes for that replicate group contain 10 hydra.

8. Observe each dish under the microscope to ensure that there are 10 hydra in each dish, and replace any hydra that are damaged in any way (eg. all buds must be attached). If not, replace immediately with 'suitable test hydra' using a new pipette.

9. Repeat steps 4-8 for the remaining two replicate groups.

Note: More than one person can distribute test hydra simultaneously, with the distribution appropriately split into replicate groups.

10. Cover the dishes and place them in the random order for that day (Section B.1.11), in the positions 1 to 21.

11. Place trays in the incubator.

Completion of this stage constitutes the start of the test (time = 0 h).

Note: Whenever test dishes are removed from the incubator maintain them at 27°C (eg. by placing them on a warming tray).

12. Observe each Petri dish at t = 2 h, after commencement of the test, under the microscope. Do not change positions of the dishes on the tray and return dishes immediately to the incubator after:

a) Counting and recording the number of hydroids, with or without buds;

b) Noting if tentacles appear clubbed or contracted; and

c) Noting any other observations that suggest the hydra are not behaving or developing normally.

Note: Observations are recorded at t = 2 h on the data sheets. To avoid observer bias, select a different replicate to observe each day. Also, commence observations with the next highest chemical concentration to that observed on the previous day (Section B.1.12).

Note: Water movement will cause temporary tentacle contraction, therefore allow the water to settle before recording observations.

Day 2

13. Dispense test solutions into appropriately labelled 100 mL vials and check the pH. If they are not within the prescribed limits, adjust accordingly using 0.02 M HNO_3 (625 µL per 500 mL) or 0.0125 M NaOH (1 g per 500 mL and diluted by 1/4).

14. When the pH range is established, dispense test solutions into appropriately labelled 45 mL vials (3 x 35 mL of each solution). Cover dispensed solutions and allow them to equilibrate to 27°C.

15. Twenty-four hours after the commencement of the test, remove the trays from the incubator, sort the test dishes into replicate groups, observe under the microscope and record as Day 1 observations.

16. After recording observations (as Day 1 observations) for a particular dish, feed each hydra in the dish.

Each hydra is fed at least 3-4 live brine shrimp nauplii, *Artemia franciscana* (Appendix C.2). The nauplii are rinsed and suspended in synthetic water and placed in each dish using a glass Pasteur pipette. Feeding is allowed to proceed *ad libitum* for at least 30 min., but 2-3 h is preferable.

17. After all hydra have been observed and fed in the 18 dishes, place the test dishes onto trays in the random order for the day (Section B.1.11), and return the trays to the appropriate position in the incubator.

18. Twenty-four hours after the commencement of the test, solutions are renewed as follows:

a) The test solution is swirled around the Petri dish to dislodge any uneaten brine shrimp and regurgitated food;

b) The solution is then tipped carefully into a second Petri dish (or cleaning dish);

c) An aliquot of the test solution (5 mL) is immediately added to cover the bottom of the test dish, the swirling process is repeated, and the solution tipped into the cleaning dish;

d) The remaining fresh solution (30 mL) is immediately added to the test dish;

e) Any hydra that are dislodged into the cleaning dish are carefully picked up with a little water using a clean pipette and returned to the test dish;

f) Any remaining brine shrimp, or other debris, in the test dish are removed by pipette, with care taken to minimise removal of test solution;

g) The cleaning dish is checked again for hydra, with any found being returned to the test dish; and

h) The solution in the cleaning dish is collected for the measurement of water parameters in each treatment after 24 h.

Note: To ensure that cross-contamination does not occur, obtain a new pipette and cleaning dish whenever a dish of lower chemical concentration is cleaned after a higher concentration.

19. Measure the physical water parameters (ie. pH, conductivity, dissolved oxygen) at the end of 24 h.

Day 3-4

20. Repeat steps 13-19 (ie. at 24 h intervals, measure and adjust synthetic water if necessary, count and record observations for the appropriate day, feed test organisms, and clean and renew test solutions).

Measure the physical water parameters and record for the appropriate day.

Note : On each day a new set of random numbers must be used for the position of each Petri dish in the incubator for the next 24 h period (Section B.1.11).

Day 5

21. Count and record observations for each test dish 96 h (4 x 24 h) after the start of the test. Do not feed hydra and do not renew test solutions.

22. Measure the physical water parameters and record as Day 4.

Test is complete.

B.1.11 Randomisation

On each day a new set of random numbers must be used for the position of each Petri dish in the incubator for the next 24 h period (see below). Randomness is an important component of the experimental design. Random distribution of hydroids is achieved via steps 4-7. The Petri dishes are randomly assigned to positions on trays each day. Since the Petri dishes have a random position on the trays, they will also have a random position in the incubator. Random numbers are obtained from a random number table or generator for each day of the test; a set of random numbers is not to be reused. When the hydra have to be observed, then the Petri dishes can be sorted into replicate groups for greater convenience. This avoids the continual changing of glass pipettes by working through the water changes from a lower to a higher chemical concentration. At the end of the water changes the Petri dishes are then again randomly placed on trays and returned to the incubator.

B.1.12 Avoiding observer bias

To avoid observer bias there are at least two observers. Each observer randomly selects a replicate group to record each day, and observations commence with the next highest chemical concentration to that which was first observed the previous day. Occasional checks should be made on the incubator performance (ie. constant temperature, light intensity, and their variation) by placing replicates in different incubators. If appreciable differences are found, then the incubator that produces the most reliable and consistent results, as outlined in Section B.1.13, should be used.

B.1.13 Acceptability of test data

The test data are considered acceptable if:

1. The recorded temperature of the incubator remains within the prescribed limits;
2. Greater than 20 healthy hydroids remain in combined control dishes at the end of the test period;
3. The recorded pH are within the prescribed limits;
4. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 27°C; and
5. The conductivity for each test solution was within 10% of the values obtained on Day 1.

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

B.2 Purple-spotted gudgeon (*Mogurnda mogurnda*) sac-fry test

B.2.1 Objective

The objective of the test is to determine the concentration of a specified chemical that shows:

a) A no-observed effect concentration (NOEC), where no statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This is compared to the 10% bounded effect concentration (BEC_{10}), where no greater than 10% effect to the test species is found (Hoekstra and Van Ewijk, 1993);

b) The lowest-observed effect concentration (LOEC), where the smallest statistical difference ($P \leq 0.05$) is found between exposed and unexposed (or control) specimens. This is compared to the minimum detectable effect concentration (MDEC), which is defined as the metal concentration at which the response became significantly ($P \leq 0.05$) lower than that of control (Ahsanullah and Williams, 1991), and

c) The median lethal concentration, where a 50% decline is found. This is measured as the LC_{50}, on the survival of *M. mogurnda* (Purple-spotted gudgeon) sac-fry over 96 h.

B.2.2 Principle of the test

Recently hatched sac-fry (< 10 h old) are exposed to a range of chemical concentrations for 96 h. Observations of any sac-fry mortality are recorded at 24 h intervals. The method is based on the 'Gudgeon embryo larval test' described by Hyne *et al.* (1996).

B.2.3 Test organism

The test species is *Mogurnda mogurnda* (Teleostomi, Eleotrididae) commonly known as the Purple-spotted or Northern trout gudgeon (Merrick and Schmida, 1984). This carnivorous species is widely distributed throughout northern Australia (Merrick and Schmida, 1984). The recommended husbandry method for *M. mogurnda* is described in Appendix D.1. Fertilised eggs are allowed to be guarded by the male parent in the aquarium for 1-2 d. They are then removed and placed in a beaker (~2 L) containing half parent tank water and half synthetic test water, and allowed to hatch at 27 ± 1°C on a warming tray in the laboratory (Appendix D.2). Gentle aeration (via an airstone) is used to simulate the male parent fanning water over the eggs to reduce the incidence of fungal spores settling. The eggs hatch after 3-4 d. Sac-fry < 10 h old are used to commence the test. Neither the embryos nor sac-fry are treated for fungus with malachite green but should be free from overt disease or gross morphological deformity. No feeding is required during the test, as the animals obtain sufficient nutrition from the attached yolk-sac.

B.2.4 Synthetic water

See Section B.1.4

B.2.5 Stock solutions

See Section B.1.5

B.2.6 Test solutions

See Section B.1.6

B.2.7 Apparatus and test equipment

See Section B.1.7

B.2.8 Test environment

See Section B.1.8

B.2.9 Data recording

See Section B.1.9

B.2.10 Test procedure

Day 1

1. Prepare the test solutions (as outlined in Section B.1.6) and leave at room temperature.
2. Isolate approximately 250 suitable test sac-fry in synthetic water in a Petri dish and leave at room temperature. A 'suitable test sac-fry' is less than 10 h old at the commencement of the test (ie. no more than 10 h have elapsed since the time of hatching). The sac-fry may be seen as a developed, hatched fry lying on the bottom of the hatching container, with a prominent yolk-sac and black-eye pigmentation visible.
3. Dispense 30 mL aliquots of each test concentration (normally 8) into 3 appropriately labelled replicate Petri dishes (ie. 3 x 30 mL for each test solution), and arrange in three replicate groups on clear plastic trays (*eg* Control Replicate 1 to X $\mu g\ L^{-1}$ on Tray 1).
4. Using a microscope and wide-mouth pipette, pick out one sac-fry from the isolated stock and place into Control Replicate 1.
5. Repeat for remaining test concentrations of replicate 1, working up in concentration, and ending with the highest concentration.
6. Discard the used pipette and select a new one.
7. Repeat steps 4-6 until all test dishes for that replicate group contain 10 sac-fry.
8. Observe each dish under the microscope to ensure that there are 10 sac-fry in each dish, and replace any sac-fry that are damaged in any way (eg. disrupted yolk etc.).
9. Repeat steps 4-8 for the remaining two replicate groups.

More than one person can distribute test sac-fry simultaneously, with the distribution appropriately split into replicate groups.

10. Cover the dishes and place them in the random order for that day (Section B.1.11), in the positions 1 to 24.
11. Place trays in the incubator.

Completion of this stage constitutes the start of the test (time = 0 h).

Note: Whenever test dishes are removed from the incubator maintain them at 27°C (eg. by placing them on a warming tray).

Day 2

12. Dispense test solutions into appropriately labelled 100 mL vials and check the pH. If they are not within the prescribed limits, adjust accordingly using 0.02 M HNO_3 (625 µL per 500 mL) or 0.0125 M NaOH (1 g per 500 mL and diluted by 1/4).

13. When the pH range is established, dispense test solutions into appropriately labelled 45 mL vials (3 x 30 mL of each solution). Cover dispensed solutions and them allow to equilibrate to 27°C.

14. Twenty-four hours after the commencement of the test, remove the trays from the incubator, sort the test dishes into replicate groups, observe under the microscope and record the following as Day 1 observations:

a) Count and record the number of live sac-fry;

b) Count and record the number of dead and/or fungoid sac-fry; and

c) Make any other observations that suggest that the sac-fry are not developing normally.

To avoid observer bias, a different set of replicates are to be observed first each day. Also, commence observations with the next highest concentration to that which was first observed the previous day (Section B.1.12).

15. After observing a dish, the test solution is renewed as follows:

a) The solution in the test dish is carefully emptied into a second Petri dish (or cleaning dish) with a gentle swirling action, tilting the dish to one side to pool the sac-fry in a small area;

b) Enough of the appropriate fresh test solution (5 mL) is immediately added to cover the bottom of the test dish, the swirling process is repeated, and the solution pipetted or carefully tipped into the cleaning dish. Keep the sac-fry submerged at all times by tilting the dish;

c) The remaining fresh solution (30 mL) is then immediately added to the test dish;

d) Any live sac-fry that are transferred to the cleaning dish at this stage are carefully put back into the test dish using a pipette;

e) Any dead sac-fry in the test dish are removed with a pipette before renewal of test solution, with care taken to minimise removal of test solution. A fresh pipette is obtained after the removal of dead sac-fry;

f) The cleaning dish is checked again for sac-fry, with any found being returned to the test dish: and

g) The solution in the cleaning dish is collected for measurement of the physical water parameters in each treatment after 24 h.

Note: To ensure that cross-contamination does not occur, obtain a new pipette and cleaning dish whenever a dish of lower chemical concentration is cleaned after a high concentration.

16. After all dishes have been observed and test solutions renewed, place dishes in the random order for that day (see below), and return trays to the incubator.

17. Measure the physical water parameters at the end of 24 h (Day 1).

Day 3-4

18. Repeat steps 12-17 (ie. at 24 h intervals count, record, renew test solutions and record the water parameters for the appropriate day).

Note: On each day a new set of random numbers must be used for the position of each Petri dish in the incubator for the next 24 h period (Section B.2.11).

Day 5

19. Count and record observations on each test dish 96 h (4 x 24 h) after the start of the test.

20. Measure the physical water parameters and record as Day 4.

B.2.11

See Section B.1.11

B.2.12

See Section B.1.12

B.2.13 Acceptability of test data

The test data are considered acceptable if:

1. The recorded temperature of the incubator remains within the prescribed limits;
2. The mean mortality of the combined control does not exceed 20%;
3. The presence of fungus on the sac-fry does not exceed 20% in any combined treatment group;
4. The recorded pH are within the prescribed limits;
5. The dissolved oxygen concentration was greater than 70% of the air saturation value throughout the test at 27°C; and
6. The conductivity for each test solution was within 10% of the values obtained on Day 1.

Note: Statistical testing should not proceed if fewer than four treatments (including Control) remain.

Appendix C Culturing Green hydra (*Hydra viridissima*) and live Brine Shrimp larvae (*Artemia franciscana*)

C.1 Culturing Green hydra

C.1.1 Primary hydra stock culture

Green hydra (*Hydra viridissima*) are cultured in the laboratory in bubble-aerated water held in 2 L glass bowls (primary stock). The bowls are loosely covered with clear polyethylene food film (eg. Glad®Wrap) so as to allow ventilation around the sides. The culture water is taken from the same batch of diluent water that is used to commence the test. The water movement caused by the gentle aeration causes most hydra to attach to the side of the bowl via the basal disc, thus reducing time taken to perform water changes.

Primary stock hydra are fed three times a week. One week prior to commenceing a test, the primary stock hydra are fed on a daily basis to achieve the maximum budding rate. Prior to commencement of this intensive feeding, hydra are observed and notes on culture health and density recorded in the primary hydra stock log book. A sample of water is then taken and the dissolved oxygen (DO_2) concentration measured and recorded as a water quality check. Hydra are then fed with newly-hatched brine shrimp nauplii (*Artemia franciscana*; Section C.2). Prior to being fed to hydra, the brine shrimp are thoroughly washed and suspended in diluent water. They are then pipetted into each primary stock bowl so that they are evenly distributed over the hydra. The hydra are allowed to feed for at least 30 min, and up to 4-5 h when possible. At the end of the feeding period, uneaten brine shrimp and regurgitated food pellets are removed by swirling the water around each bowl and emptying it into a second cleaning dish (eg. 4 L plastic ice-cream container). More diluent water is added and the procedure repeated until each bowl is free of brine shrimp. The bowls are then re-filled with approximately 1.5 L of clean water. Any hydra removed by the process are pipetted back into the glass bowl containing the fresh water. This process is referred to as a 'rinse' clean.

Stock bowls are cleaned at least twice weekly by performing a 'scrub' clean. After observations are made and recorded, samples taken to measure DO_2 levels, the excess water is carefully decanted away, ensuring that minimal hydra are lost. If necessary, the old water can be decanted into a cleaning dish so that enough hydra can be retained during cleaning. The bowls are then cleaned by gently pushing with the fingers the attached hydra away from the sides of the bowl, and into a cleaning dish. Clean hands, or hands covered by gloves should be used to perform this procedure. The detached hydra are allowed to settle into a corner of the cleaning dish by slightly tipping the dish. Using a glass Pasteur pipette, the

hydra can then be transferred to a clean glass bowl containing fresh water. Bowls are washed by analytical-grade dishwasher (eg. Gallay Laboratory 999). Immediately prior to use, the bowls are rinsed with fresh diluent water.

C.1.2 Secondary hydra stock culture

A secondary stock of hydra are maintained in tap-water filled aquaria in a separate location, as a precaution against contamination or accidents. The aquaria are maintained as 'community' tanks, with 3-4 small fish (eg. *Ambassis sp., Pseudomugil sp.*) and freshwater snails.

The secondary hydra stock are fed daily with brine shrimp, and the aquaria cleaned at least once a week. Excess hydra are gently pushed away from the sides of the aquaria and siphoned out. A third of the tank water should also be siphoned out and replaced as part of the cleaning process. Cladocera (*Moinodaphnia macleayi*) are fed at least once a week to the primary and secondary hydra cultures as a natural diet supplement.

C.1.3 Sexual reproduction in hydra cultures

Periodically, hydra are observed to reproduce sexually, making it difficult to maintain an isogenic population. This could be related to fouling of the holding water and fungal growth on the uneaten brine shrimp. The frequency with which sexual reproduction occurs can sometimes be reduced by increasing the rate of feeding and cleaning of the primary cultures. If fungal contamination is observed at any time, the bowls can be given a 'rinse' clean.

C.2 Culturing of live Brine Shrimp larvae

C.2.1 Hatching brine shrimp larvae

Brine shrimp (*Artemia franciscana*) are used as food for many types of aquatic organisms, including larval fish and hydra. Brine shrimp can be cultured in a variety of containers to give an uninterrupted supply of nauplii (juvenile brine shrimp). The most appropriate type of culture containers are conical flasks (conical 1 L separation funnels are ideal) which, when inverted with the neck downwards, can be bubble-aerated from the bottom with oil-free compressed air. An 800 mL salt solution is made by dissolving 30 g of coarse rock salt, or sea salt, in 800mL of warm water (28°C). After the salt is fully dissolved, one teaspoon (~5 g) of commercially harvested, dried brine shrimp cysts are added. Vigorous bubbling from the bottom of the container prevents eggs from settling. Brine shrimp eggs will hatch in 18-24 h at an incubation temperature of 28°C and in an outside shaded position. At lower temperatures, hatching is delayed. On cloudy days the culture may need to be directly illuminated by a fluorescent lamp, as hatching is light dependent.

C.2.2 Harvesting brine shrimp larvae

To harvest the newly-hatched nauplii, the compressed air is turned off 24 h after adding the eggs to allow the nauplii to settle and empty egg shells to float. After ~5 min, the nauplii are strained through a fine nylon mesh net which is able to retain the nauplii, and then washed with the test dilution water. The washed nauplii are then re-suspended in a small volume of dilution water (about 5 mL) and placed in a small beaker or Petri dish which is inclined at an angle of approximately 45° towards the light. Live nauplii will concentrate in the upper layer, while the unhatched cysts will remain on the bottom of the container. The upper layer, containing live nauplii, is then collected for feeding. A Pasteur pipette or syringe is used to collect and distribute the nauplii.

Appendix D Recommended husbandry of Purple-spotted gudgeon (*Mogurnda mogurnda*) and method of isolating their sac-fry

D.1 Recommended husbandry method of Purple-spotted gudgeon

D.1.1 Collection and acclimation

Purple-spotted gudgeon (*Mogurnda mogurnda*) are collected from local waterways within the Magela Creek system of the Alligator Rivers Region, NT, Australia. Fish are captured either by baited fish traps or by fine meshed dip nets or seine nets, and are brought back to the aquaculture facilities at the Environmental Research Institute of the Supervising Scientist. Initially they are placed in either 80 L or 200 L aquaria, where the number of fish in each aquarium is determined by the size of the fish. Observations are then made for a nominal period to ascertain fish health and acclimation to laboratory conditions, and also to determine the sex of the fish based on physical appearance of the papilla. Once the sex has been determined, the fish are divided into breeding groups, consisting of one male and one to three females per aquarium. Further observations are then carried out to assess the breeding groups for fecundity, fertility and embryo hatchability to avoid any site-specific trait interfering with a test.

D.1.2 Aquaria layout

The aquaria are set up in a row within a shaded aquaculture area, running along an east-west aspect. Washed gravel covers the bottom of the tanks, and a local green weed grows near the surface of the water providing refuge. Six washed black plastic plant pots with a diameter of 23 cm are placed in the tanks. Gravel or small stones are placed inside the pots to anchor them, and the opening of each pot is directed towards the front of the viewing area to assist observation. The pots provide a 'cave' refuge for the fish, and also a spawning surface.

It is advantageous to have at least 4-6 breeding aquaria set up and producing sac-fry so that a toxicity test can be commensed when needed. Aquaria used for the fish are filled initially with tap water, and then 'modified' for the production of test embryos by the addition of either chilled deionised water, natural creek water, or synthetic water (ie. chilled low conductivity water representing a storm event). The aquaria are located in a shaded aquaculture area outside the main testing laboratory; the water temperature in the aquaria during the Dry season ranges from 24-28^{o}C, whereas during the build-up and subsequent Wet season it ranges from 26-32^{o}C. A cooler temperature is maintained during the warmer

months by the addition of chilled water during a water change. Undergravel filters provide aeration coupled with a natural photoperiod.

D.1.3 Fish feeding and aquaria maintenance

Fish are fed once daily on a varied diet consisting of 'commercial fish pellet' (Aristo Pet high protein fish pellets) supplemented with live food when possible (eg tadpoles, water boatmen etc). It has been observed that such a diet is adequate to provide sufficient nutrition to the breeding fish and enable the continuous production of embryos for weeks at a time. In addition, it has been observed that the quality of the water in the aquaria can be maintained at a higher level with less fouling when using such food. Live food, such as tadpoles, can be captured and placed with the fish, allowing the fish to continue eating *ad libitum*.

The aquaria are cleaned on a fortnightly basis (or more frequently if required) using a wide mouth vacuum siphon. The gravel is disturbed, allowing trapped leftover food, faeces and any other debris to be removed. To ensure fish are not subject to undue stress, a quarter to a third water change is performed, and the water replaced either with chilled low conductivity water or tap water at ambient temperature.

D.1.4 Courtship and spawning

Gudgeon breeding is variable, however it is possible to predict an approximate time a batch of eggs will be produced, based on careful observation of both behaviour and physical characteristics of a pair of fish (ie courtship behaviour accompanied by distinct golden colouration on the abdomen of the breeding female, and swelling and protrusion of both male and female papilla).

The male will select a spawning site (eg. back of thermometer, rock, side of tank), and the female lays a batch of eggs while the male fertilises them. Each day prior to feeding, the tanks are carefully observed for the presence of newly spawned eggs with the aid of a torch. The eggs are tubular in shape, have transparent cases, and are generally laid in circular patches of various sizes depending on the size of the breeding female. The egg batches range in size from 300–1000 eggs. The eggs are left in the aquarium to be guarded by the male parent fish for 24–48 h after being laid. They are then removed from the breeding aquarium and either kept and reared as future in-house breeding stock, or are placed in a 2 L beaker containing half parent tank water and half test diluent water and allowed to hatch under laboratory conditions for use in a toxicity test (Appendix B).

If a breeding group cease spawning, the fish can be swapped into different tanks with different combinations of groups of females. Alternatively, spawning can be delayed in a tank by placing a partition in it such that the male is isolated from the breeding females. After the partition is removed, it has been observed that spawning recommences within

1–2 d. This is beneficial for obtaining fish early in the week. If there is excessive disturbance or pedestrian traffic around the aquaria, opaque perspex screens can be positioned around an area of an aquarium that is being used for spawning.

D.2 Isolation of Purple-spotted gudgeon sac-fry

When a batch of eggs is produced, they are left in the parent tank for 24–48 h allowing the male parent fish to guard them. Infrequently the eggs may be eaten before they can be removed, however it is noted that this is the exception rather than the rule, and may be due to the presence of excessive numbers of water mites (eg Suborder Oribatida) and microcrustacea in the breeding aquaria which invade and feed on the egg mass. To reduce the numbers of such fauna, a small Black-striped rainbowfish (*Melanotaenia nigrans*) can be placed in a breeding aquaria.

After 24–48 h development in the parent tank, the developing embryos are carefully removed by placing the pot or rock etc, into a 2 L beaker containing half parent tank water and half diluent water, ensuring that the temperature of this water is ± 1°C of the parent tank water. If the eggs are laid on a surface such as the wall of a pot, eggs can be removed for observation by carefully sliding a glass cover slip under the egg mass and moving it forward until the edge has some eggs attached to it. The cover slip with the eggs is transferred to a Petri dish with enough water to cover them while observations are made. The batch of developing embryos is then placed on a warming tray set at 27 ± 1°C in the laboratory to continue development. They are observed for deformities, viability or water mites etc. An airstone is positioned beneath the egg batch such that a gentle stream of bubbles passes upward over the surface of the eggs, simulating the fanning action of the male parent over the eggs to keep fungal spores from settling. The beaker is loosely covered with Glad®Wrap to stop dust etc. Frequent daily observations are made, ensuring minimal disturbance until hatching occurs. Half water changes are performed using test diluent water to ensure fouling does not occur. It takes approximately 10 h for the entire batch of eggs to hatch into sac-fry. To determine the age of the embryos they are observed under a stereo microscope while still covered with water. After all the eggs have hatched (or at least sufficient numbers to enable a test to be commenced), they are carefully isolated into Petri dishes using a glass Pasteur pipette with an internal diameter at least 2 mm. Enough sac-fry are placed in each Petri dish so that there are enough for each replicate to be started. Any damaged sac-fry are discarded and sacrificed by exposing them to a 1 g L^{-1} solution of the anaesthetic, MS222.

Appendix E Maintaining constant pH in toxicity studies

E.1 Rationale

pH is an important factor affecting the toxicity of Cu and U to freshwater organisms. pH may affect metal toxicity directly by affecting metal uptake or indirectly by affecting the chemical speciation of the dissolved metal. The inability to distinguish between these two mechanisms has led to much confusion in the literature dealing with the effects of pH on metal toxicity. Previous studies have suggested Cu and U toxicity decrease at lower pH (Horne and Dunson, 1995; Franklin *et al.*, 1998), while others have reported the opposite effect (Schubauer-Berigan *et al.*, 1993; Erickson *et al.*, 1996). The variability in results has prevented a definitive metal-pH relationship from being established for Cu and U. Hence, this study identified the importance of isolating and assessing the effects of pH, at constant hardness and alkalinity, on the toxicity of Cu and U to *H. viridissima* (Green hydra, population growth).

E.2 Methodology

Toxicity testing materials and procedures are detailed in Chapter 2. Only specific modifications made to these standard protocols are mentioned here.

E.2.1 Selection of pH values

Regional water quality information was gathered from Northern Territory Water Resources and the Environmental Research Institute of the Supervising Scientist to determine a relevant pH range for tropical Australian freshwater systems (Refer Figure 3.1; Table 3.1).The pH values of 5.0, 6.0 and 7.0 were selected to represent the general range found in tropical freshwaters of Northern Territory, and also to contribute applicable information to Australian water quality guidelines.

E.2.2 Acclimation of *H. viridissima*

Prior to commencing toxicity tests, test organisms were acclimated to the selected pH conditions, so as to eliminate adverse effects of transferral from stock culture water to the test solution. A laboratory stock of *H. viridissima* existing in pH 6.0 synthetic Magela creek water (Appendix C), was divided into three groups, with one group remaining in pH 6.0 synthetic water. The other two groups were acclimated in synthetic water to pH 5.0 and 7.0 by adjusting pH gradually. The frequency of feeding and cleaning the culture was increased to once a day so that population growth was optimised. Each time the water was renewed a quarter of the volume was replaced with pH 5.0 or pH 7.0 water, so that after 96 h the medium was at the required pHs. If the organism appeared stressed, (ie. clubbed or

reproducting sexually) the process of altering the pH was ceased until the animals appeared healthy again.

At pH 7.0, *H. viridissima* were found to reproduce at the same rate as hydra exposed to pH 6.0. However, hydra did not survive more than two weeks in synthetic water at pH 5.0. Consequently, pH 5.5 was chosen as a substitute for pH 5.0 as it reflects the difference between 6.0 and 7.0 on the pH log-scale, and because it was more likely that hydra could tolerate it. A group of hydra were collected from the pH 6.0 stock culture, and acclimated to pH 5.5 by gradually lowering the pH. Hydra at pH 5.5 were found to reproduce at the same rate as hydra in pH 6.0 and 7.0 synthetic water.

E.2.3 Isolating pH effects

A series of tests were conducted to ensure toxicity test endpoints were not significantly ($P > 0.5$) affected by an increase or decrease in pH. It was important to ensure that at pH 5.5 and 7.0 *H. viridissima* population growth did not differ significantly ($P > 0.5$) from the responses at pH 6.0 (baseline).

To achieve pH 5.5, 6.0 and 7.0, the diluent water was adjusted using 0.2 M HNO_3 or 0.05 M NaOH. To compensate for the expected shift in pH, the measured pHs prior to solution renewal were 5.4, 6.0 and 7.1.

It was observed that the measured pH of 'pH 5.5' and 'pH 7.0' solutions deviated significantly (> 0.5 pH units) from nominal values within 24 h. It was observed that the 'pH 5.5' solution had increased to ~ pH 6.0 and the 'pH 7.0' solution dropped to ~ pH 6.5. A pH drift of ± 0.5 units could not be accepted as a valid indicator of the effect of pH on Cu and U toxicity. Thus, it was necessary to develop a technique that would reduce the shift in pH.

E.2.4 Changes to test protocols to allow water parameter manipulation

Two techniques were investigated to stabilize pH to within 0.3 units of the nominal pH of treatment solutions:

Repeated pH adjustment

The first technique involved adjusting the pH of the treatment solution twice within the 24 h renewal period, instead of once as stated in the test protocol (Appendix B). Initially, it was thought that the treatment solution could be adjusted once prior to solution renewal and then again approximately six hours later as shown by Franklin *et al.* (1998). However, this proved impractical as the test organisms were contained in Petri dishes filled with 30 mL of treatment solution, creating a very shallow medium. Adjusting the pH of 30 mL solutions was perceived to potentially compromise organism health, where by the test organisms could

be disturbed by handling, stirring of the mixture to achieve a uniform pH, and/or by directly receiving the drop of acid/base when altering the pH.

In an attempt to better characterize the pH fluctuation, the pH was measured over 24 h to detect when the greatest pH change occurred. Eight Petri dishes containing 30 mL of synthetic water at pH 5.5 were each allocated to one of eight times (0, 1, 2, 4, 6, 8, 22 and 24 h). At each time interval the appropriate dish was emptied and the pH of the solution recorded. The solution was not returned to the Petri dish. It was found that the greatest shift in pH occurred between 0 h and 2 h. Noting this, the pH of the treatment solution was adjusted and allowed to equilibrate before making the second adjustment. The test protocol was then followed as outlined in Appendix B. In brief, 30 mL aliquots were poured into three replicate Petri dishes for each pH of interest. Ten hydra were then added to each dish. After 24 h, the 30 mL replicates were combined according to respective pH, and the pH recorded.

Increased test solution volume

The second technique involved increasing the volume of the treatment solution to provide a greater buffering capacity. Plastic specimen jars (200 mL with two holes drilled in lids) were selected to ensure manipulation of test organisms under the microscope remained possible. Two replicate jars were filled with 150 mL of synthetic water and allocated ten hydra each. The pH was recorded at specific time intervals (0, 1, 2, 4, 6, 8, 22, 24 h) without removing the solution from the jar or disturbing the animals. After measuring the pH at each time interval the jars were returned to the incubator.

E.3 Results

E.3.1 Repeated pH adjustment

Prior to trialling the technique of adjusting the pH twice within each 24 h period of a toxicity test, the shift in pH over 24 h was observed. After 24 h the mean pH of the diluent water increased from 5.46 to 6.01 (Table E.1). It was also observed that the pH deviated beyond the acceptable range of 5.5 ± 0.3 between 0 h and 2 hr. Within the first 2 h period, the mean pH increased from 5.46 to 5.83 (Table E.1).

Table E.1: Measured pH of duplicated 30 mL volumes of pH 5.5 synthetic water recorded at specific time intervals over a 24 hour period.

Time intervals (h)	Rep1 Measured pH	Rep2 Measured pH
0	5.46	5.46
1	5.64	5.56
2	5.85	5.80
4	5.85	5.81
6	5.86	5.90
8	5.90	5.95
22	6.00	5.96
24	6.03	5.99

When *H. viridissima* were included in the toxicity test, the pH of treatment solutions also drifted from nominal pH values. The measured pH did not deviate beyond 6.0 ± 0.3, but the pH did drift beyond 5.5 ± 0.3 and 7.0 ± 0.3 (Table E.2). Where a pH shift within ± 0.3 units over 24 h is acceptable.

Table E.2: Measured pH of duplicated treatments at 0 h and 24 h which were pH-adjusted twice prior to the inclusion of *H. viridissima.*

Nominal pH	Replicate 1		Replicate 2	
	Measured pH at 0 h	Measured pH at 24 h	Measured pH at 0 h	Measured pH at 24 h
5.5	5.41	5.85	5.37	5.84
6.0	5.93	6.01	6.02	6.14
7.0	7.12	6.57	7.09	6.56

E.3.2 Effect of solution volume on pH shift

The pH of 150 mL treatment solutions shifted beyond 5.5 ± 0.3 over 24 h (Table E.3). The mean pH of synthetic water increased from 5.56 to 5.91.

Table E.3: Measured pH of duplicated 150 mL volumes of pH 5.5 synthetic water recorded at specific time intervals over a 24 hour period.

Time intervals (h)	Rep1 Measured pH	Rep2 Measured pH
0	5.56	5.56
1	5.62	5.60
2	5.64	5.62
4	5.71	5.64
6	5.73	5.64
8	5.80	5.78
22	5.86	5.80
24	5.92	5.89

E.4 Discussion

To accurately assess the effects of pH on the toxicity of Cu and U to freshwater organisms it was necessary to acclimate the test organisms to the selected pH conditions. Acclimation ensures there is no adverse effects following transferral from stock culture water to the test solution. A laboratory stock of *H. viridissima* was maintained in synthetic water with a mean pH of 6.0 (Appendix C). This population was divided, and the organisms acclimated to pH 5.0 and 7.0 synthetic water. Hydra successfully acclimated to pH 7.0 water, but did not survive more than two weeks in pH 5.0 water. A study by Hyne *et al.* (1992) on the pH-dependence of U toxicity to *H. viridissima*, reported the pH-range of 5.0-7.0 to not significantly affect hydra population growth in the absence of U. It could be suggested that the organic component of the natural creek water, used by Hyne *et al.* (1992), buffered the pH and consequently improved the survival and reproduction of the test organisms. Due to pH 5.0 proving lethal to hydra, pH 5.5 was selected, as it would reflect the difference between 6.0 and 7.0 on the pH log-scale, and because it was more likely that hydra would tolerate it.

In preliminary tests to eliminate factors that may confound the effects of pH it was discovered that the pH drifted substantially (*ie.* > 0.3 pH units) from the nominal value. The standard method of adjusting the synthetic media at the commencement of the toxicity test did not maintain the pH over 24 h. Franklin *et al.* (1998) solved this problem by adjusting the pH twice. However, this method could not be applied to the experimental design in this study, as the test organisms would be disturbed in the shallow media (*ie.* 30 mL in a Petri dish). It was found that the greatest change in pH over 24 h was within the first two hours. Despite adjusting the pH twice prior to solution renewal, the pH was not maintained within a

reasonable range of 5.5 ± 0.3 or 7.0 ± 0.3, with or without test organisms included in the solution. The observed change in pH could be explained by the low buffering capacity of the water, where the CO_2 in the air, and that respired from test organisms, produces alkaline metal carbonates in the water which cause the pH to equilibrate toward pH 6.0 (*ie.* pH of unmanipulated synthetic test water). Using a larger volume of solution proved unsuccessful in providing a larger buffering capacity to reduce this process.

The use of a pH buffer to maintain pH was decided against due to the complexing nature of buffers and the possible interference with the test animals. However, later work in this study demonstrated 0.25 M MES biological buffer (2-morpholinoethanesulphonic acid) to be successful in maintaining pH at 6.5 when assessing the effects of alkalinity on the toxicity of Cu and U to *H. viridissima* (Chapter 4). Thus, it may be possible to use MES buffer to assess the effects of pH on the toxicity of Cu and U to *H. viridissima*.

E.5 Conclusions

It is recognised that pH is an important factor affecting metal availability and toxicity to aquatic organisms. In the process of investigating the effect of pH, it became apparent that constraining the pH by methods of adjusting the solution twice prior to renewal and using a larger volume made no difference to the pH drifting towards pH 6.0 (ie. pH 6.0 being the typical pH of the synthetic water used in this experimental design). MES biological buffer may however, constrain the pH to a reasonable range around pH ~5.5-6.5. If so, the buffer could be used in the current method to accurately assess the influence of pH on the toxicity and bioavailability of heavy metals to freshwater organisms.

In this study, it was observed that the pH drifted beyond a reasonable range of 5.5 ± 0.3 and 7.0 ± 0.3 over 24 h. Such pH drifts negate the purpose of investigating a pH effect, and may significantly alter metal speciation, and thus, metal bioavailability. Subsequently, this project investigated the influence of true water hardness and alkalinity on the toxicity of U and Cu to *H. viridissima* and *M. mogurnda*. These parameters are of particular interest as the results from the toxicity tests will clearly separate the effects of the two water parameters, which have been confounded in previous work. All tests were conducted at pH 6.0.

These findings should be considered in further investigations of pH influencing U and Cu toxicity to *H.viridissima* and *M.mogurnda* sac-fry, and the possible modification of the current protocol to accommodate manipulative experimentation and buffers.

Appendix F Raw data for final day toxicity test results

Table F.1: Number of *Hydra viridissima* hydroids after 96 h exposure to Cu at three hardness levels.

Hardness[a] (mg L^{-1})	Nominal [Cu] (µg L^{-1})	Test 1			Test 2			Test 3			Test 4		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10	Rep 11	Rep 12
6.6	0.7	37	23	27	37	36	37	30	31	32			
	1	30	33	24	35	29	31	32	29	31			
	2	22	21	19	33	24	20	31	25	23			
	3	21	18	19	20	19	21	26	28	34			
	4				21	20	20	23	20	28			
	5	21	18	16	20	20	19	22	18	19			
	6							16 / 11[b]	15 / 10[b]	15 / 17[b]			
	8	8	7	4	10	0	4						
	10	0	0	1	4	1	0						
	15	0	0	0									
165	0.7	32	34	33	52	45	39	33	28	37	33	27	34
	1	37	42	33	46	41	43						
	2	31	37	27	37	35	31	24	28	31	29	27	31
	3							34	25	28	22 / 25[b]	21 / 28[b]	19 / 21[b]
	4	27	25	24	30	33	29				21	19	19
	5							8	13	12	19 / 22[b]	19 / 20[b]	19 / 21[b]
	6	17	21	16	31	17	27	13	11	12	16	18	16
	8							6	10	11			
	10	12	4	9	9	8	11	3	2	3			
	12	3	10	3	1	4	3						
	15	0	0	1	0	0	0						
	20							0	0	0			

Table F.1 Cont'd

Hardness[a] (mg L^{-1})	Nominal [Cu] (µg L^{-1})	Test 1			Test 2			Test 3			Test 4		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10	Rep 11	Rep 12
330	0.7	37	41	42	39	35	26	34	32	31			
	1				36	28	30	30	18	36			
	2	29	28	36				29	30	28			
	3				26	30	24	26	31	20			
	4	16	25	23	17	22	21	19	25	25			
	5				18	11	16	20	23	22			
	6	15	15	19	18	18	15	15	15	21			
	8	10	15	13	7	7	1	8	12	12			
	10	2	2	4									
	12	0	0	0									
	15				0	0	0						

[a] Hardness measured as mg $CaCO_3$ L^{-1}.

[b] Value corresponds to a second replicate at respective metal concentration (*ie.* the test contained two replicates at the same concentration).

Table F.2: Number of surviving *Mogurnda mogurnda* sac-fry after 96 h exposure to Cu at three hardness levels.

Hardness[a] (mg L^{-1})	Nominal [Cu] ($\mu g\ L^{-1}$)	Test 1		Test 2				Test 3	
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8
6.6	5	10	10	10	10	10	10	10	10
	10	10	10	10	9	8	10		
	15	10	10	8	4	8	8	9	6
	17					7	5	3	3
	20	6	7	1	1	4	4	1	0
	25	5	6	0	0	2	3		
	30	1	2	0	0	0	0	0	0
	50	0	0	0	0				
165	0.7	10	10	10	10	10	9	10	10
	5	10	10					10	10
	10	10	10			7	8		
	15	10	10	10	9				
	20	10	10					5	6
	25	10	9	8	9				
	30	5	9	10	8	1	2	1	3
	40					2	1	2	2
	50	4	8	1	1	0	0		
	60					0	2	1	0
	70			1	0	0	0		
	90			0	0	0	0		
	110			0	0				

Table F.2 Cont'd

Hardness[a] (mg L^{-1})	Nominal [Cu] (µg L^{-1})	Test 1		Test 2				Test 3	
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8
330	0.7	10	10	10	10	9	10	10	9
	5	9	10					10	10
	10	9	9			6	9		
	15	10	10	8	9				
	20	10	10					3	3
	25	10	10	8	6	1	2		
	30	9	10	6	6	3	1	1	4
	40					1	1	3	3
	50	7	6	1	0	2	4		
	60					1	0	2	0
	70			0	0	1	0		
	90			0	0				
	110			0	0				

[a] Hardness measured as mg $CaCO_3$ L^{-1}.

Table F.3: Number of *Hydra viridissima* hydroids after 96 h exposure to U at three hardness levels.

Hardness[a] (mg L^{-1})	Nominal [U][b] (µg L^{-1})	Test 1			Test 2			Test 3			Test 4		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10	Rep 11	Rep 12
6.6	0.1	24	33	35	23	25	25	30	28	30	39	39	36
	5	28	34	31									
	10	27	28	29									
	30	26	28	24	25	23	27						
	50	29	27	29				22	24	23			
	70				20	21	26	21	21	20	32	45	31
	100	24	21	20	20	20	21	19	22	23			
	110										23 / 24[b]	25 / 20[b]	22 / 22[b]
	125				9	5	9	12	18	13	21	19	18
	140										19 /19[b]	22 / 21[b]	20 / 13[b]
	150				3	2	0	16	8	1			
	175				2	1	1	1	1	0			
	200	0	0	0	0	0	1	1	0	0			
	400	0	0	0									
165[c]	0.1	23	24	24	35	18	22	23	21	21	21	20	19
	25	21	22	24									
	50	23	22	23									
	75				36	26	29	22	21	21	20	18	18
	100	20	21	21	23	25	31	18	18	19	18	20	19
	130				21	26	21	19	19	20	17	18	18
	150							19	18	19	14	20	18
	170				18	15	21						
	180							14	13	14	9	14	6
	200	14	12	14	5	11	7				2	6	1
	225							3	0	3	1	0	0

Table F.3 Cont'd

Hardness[a] (mg L^{-1})	Nominal [U][c] (µg L^{-1})	Test 1			Test 2			Test 3			Test 4		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10	Rep 11	Rep 12
165	250				0	2	0	0	0	1			
	300				0	0	0						
	400	0	0	0									
	600	0	0	0									
	1000	0	0	0									
330	0.1	39	37	42	30	36	42	45	43	50	40	35	39
	25				28	39	42						
	50	40	31	39									
	75	31	31	35	32	40	34	44	46	44			
	100	27	28	28	32	35	31				39	38	25
	125				27	30	30	44	39	44			
	150				22	30	26	40	39	47			
	175							36	38	28	30	24	24
	200				16	20	20	29	36	39	22	21	20
	225							31	32	26	21	10	16
	235										20	22	16
	245										17	12	13
	250	4	2	0	2	11	6						
	300							9	11	3	4	2	0
	500	0	0	0									
	750	0	0	0									

[a] Hardness measured as mg $CaCO_3$ L^{-1}.

[b] Value corresponds to a second replicate at respective metal concentration (*ie.* the test contained two replicates at the same concentration).

[c] Uranium (U) concentration is expressed as uranyl (*ie.* UO_2).

Table F.4: Number of surviving *Mogurnda mogurnda* sac-fry after 96 h exposure to U at three hardness levels.

Hardness[a] (mg L^{-1})	Nominal [U][b] (µg L^{-1})	First investigation						Second investigation					
		Test 1		Test 2		Test 3		Test 1		Test 2		Test 3	
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
6.6	0.1	9	10	10	10	10	10	10	9	10	10	10	10
	500	10	9	10	10	10	10	10	10	10	10	10	10
	1000	9	10	10	10	10	10	9	8	10	9	10	10
	1200	10	10	10	10	10	10	9	5	10	10	10	10
	1400	8	10	10	10	9	10	9	6	9	9	10	10
	1600	6	3	8	7	8	7	5	1	8	10	10	10
	1800	3	5	5	2	6	6	1	0	4	8	6	7
	2000	3	2	0	1	0	2	1	0	3	3	7	4
165	0.1	9	10	10	10	10	10	10	10	10	10	10	10
	500	10	9	10	10	10	10	10	10	10	10	10	10
	1000	5	6	10	10	10	10	10	10	10	9	9	10
	1200	4	1	8	7	5	9	8	8	10	8	10	10
	1400	0	2	0	2	3	4	5	2	8	6	9	9
	1600	1	3	0	0	0	0	0	1	5	6	9	8
	1800	0	0	0	0	0	0	0	1	1	4	6	7
	2000	0	0	0	0	0	0	0	0	0	1	2	6

Table F.4 Cont'd

Hardness[a] (mg L^{-1})	Nominal [U][b] (µg L^{-1})	First investigation						Second investigation					
		Test 1		Test 2		Test 3		Test 1		Test 2		Test 3	
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6
350	0.1	8	9	10	10	10	10	10	10	10	10	10	10
	500	10	10	10	10	10	10	10	10	10	10	10	10
	1000	7	8	10	7	10	10	9	7	8	9	10	10
	1200	6	5	2	3	8	5	9	8	7	8	10	10
	1400	5	2	1	1	2	4	7	8	6	8	9	10
	1600	3	2	0	0	0	0	1	2	2	7	7	6
	1800	6	5	0	0	0	0	0	0	2	2	3	5
	2000	0	0	0	0	0	0	0	0	0	1	1	1

[a] Hardness measured as mg $CaCO_3$ L^{-1}.

[b] Uranium (U) concentration is expressed as uranyl (*ie.* UO_2).

Table F.5: Number of *Hydra viridissima* hydroids after 96 h exposure to Cu at two alkalinity levels.

Alkalinity[a] (mg L^{-1})	Nominal [Cu] (µg L^{-1})	Test 1			Test 2			Test 3		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9
4	0.7	37	23	27	37	36	37	30	31	32
	1	30	33	24	35	29	31	32	29	31
	2	22	21	19	33	24	20	31	25	23
	3	21	18	19	20	19	21	26	28	34
	4				21	20	20	23	20	28
	5	21	18	16	20	20	19	22	18	19
	6							16 / 11[b]	15 / 10[b]	15 / 17[b]
	8	8	7	4	10	0	4			
	10	0	0	1	4	1	0			
	15	0	0	0						
102	0.7	44	46	45	33	33	35			
	2	41	38	43	27	27	25			
	3	36	29	34	28	25	27			
	4	30	27	38	20	18	19			
	5	24	26	23	19	20	21			
	8	12	16	10	8	10	8			
	12	5	4	3	0	0	0			
	20	0	0	0	0	0	0			

[a] Alkalinity measured as mg $CaCO_3$ L^{-1}.

[b] Value corresponds to a second replicate at respective metal concentration (*ie.* the test contained two replicates at the same concentration).

Table F.6: Number of *Hydra viridissima* hydroids after 96 h exposure to U at two alkalinity levels.

Alkalinity[a] (mg L^{-1})	Nominal [U][b] (µg L^{-1})	Test 1	Test 2					Test 3			Test 4		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10	Rep 11	Rep 12
4	0.1	24	33	35	23	25	25	30	28	30	39	39	36
	5	28	34	31									
	10	27	28	29									
	30	28	28	24	25	23	27						
	50	29	27	29				22	24	23			
	70				20	21	26	21	21	20	32	45	31
	100	24	21	20	20	20	21	19	22	23			
	110										23 / 24[b]	25 / 20[b]	22 / 22[b]
	125				9	5	9	12	18	13	21	19	18
	140										19 /19[b]	22 / 21[b]	20 / 13[b]
	150				3	2	0	16	8	1			
	175				2	1	1	1	1	0			
	200		0	0	0	0	1	1	0	0			
	400	0	0	0									

Table F.6

Alkalinity[a] (mg L^{-1})	Nominal [U][c] (µg L^{-1})	Test 1			Test 2			Test 3			Test 4		
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10	Rep 11	Rep 12
102	0.1	24	23	30	26	28	31	36	45	41			
	20							39	34	39			
	50	26	24	23	21	24	23						
	75				20	18	26	43	28	40			
	100	47	19	23	23	0	9	43	37	37			
	125				23	19	15	36	29	28			
	150	16	11	13	19	19	9	27	22	28			
	170	14	6	11	19	2	3	23	26	28			
	200	0	1	0	1	5	2	10	11	13			
	250	0	0	0									
	400	0	0	0									

[a] Alkalinity measured as mg $CaCO_3$ L^{-1}.

[b] Value corresponds to a second replicate at respective metal concentration (*ie.* the test contained two replicates at the same concentration).

[c] Uranium (U) concentration is expressed as uranyl (*ie.* UO_2).

Appendix G Summary data for concentration-response curves

Table G.1 Percent population growth response of *Hydra viridissima* to a range of Cu concentrations at three hardness levels.

Nominal Cu concentration (µg L^{-1})	6.6 mg L^{-1} hardness (as $CaCO_3$)			165 mg L^{-1} hardness (as $CaCO_3$)			330 mg L^{-1} hardness (as $CaCO_3$)		
	Measured Cu concentration (µg L^{-1})	% Population growth	95% C.I.	Measured Cu concentration (µg L^{-1})	% Population growth	95% C.I.	Measured Cu concentration (µg L^{-1})	% Population growth	95% C.I.
0.7	0.7	100	0	0.7	100	0	0.7	100	0
1	0.9	91	5.3	0.9	97	4.3	0.9	86	13.2
2	1.9	76	10.9	1.7	86	5.7	1.8	82	7.0
3	2.4	69	11.7	2.4	78	10.3	2.4	77	11.6
4	3.0	64	8.9	3.0	66	4.4	4.2	60	7.7
5	3.8	57	3.2	4.0	54	10.8	5.2	54	11.1
6	5.2	43	6.4	5.2	48	5.8	5.5	47	4.1
8	7.0	16	7.7	7.0	28	10.4	7.1	26	7.1
10	8.9	3	3.4	9.0	17	6.2	8.2	6.6	2.9
12				10.0	10	5.9	10.0	0	0
15	13.5	0	0	14.0	0.5	0.9	12.1	0	0
20				18.0	0	0			

Table G.2 Percent survival response of *Mogurnda mogurnda* to a range of Cu concentrations at three hardness levels.

Nominal Cu concentration (µg L^{-1})	6.6 mg L^{-1} hardness (as $CaCO_3$)			165 mg L^{-1} hardness (as $CaCO_3$)			330 mg L^{-1} hardness (as $CaCO_3$)		
	Measured Cu concentration (µg L^{-1})	% Survival	95% C.I.	Measured Cu concentration (µg L^{-1})	% Survival	95% C.I.	Measured Cu concentration (µg L^{-1})	% Survival	95% C.I.
0.7	0.7	100	0	0.7	99	2.4	0.7	98	3.2
5	4.3	100	0	4.5	100	0	5.4	98	4.9
10	6.8	95	6.7	7.5	88	14.7	8.4	83	14.7
15	11.4	79	14.1	9.1	98	4.9	12.0	93	9.4
17	11.8	45	18.8						
20	15.1	30	18.1	14.1	78	25.8	15.0	65	39.6
25	19.2	27	20.0	20.0	90	8.0	19.7	62	31.4
30	21.5	3.8	5.2	23.1	49	25.5	24.4	50	23.4
40				33.1	18	4.9	31.9	20	11.3
50	33.0	0	0	39.2	23	25.1	41.9	33	22.4
60				50.7	8	9.4	50.6	8	9.4
70				58.7	3	4.9	55.9	3	4.9
90				81.0	0	0	80.4	0	0
110				100.0	0	0	100	0	0

Table G.3 Percent population growth response of *Hydra viridissima* to a range of U concentrations at three hardness levels.

6.6 mg L^{-1} hardness (as $CaCO_3$)				165 mg L^{-1} hardness (as $CaCO_3$)				330 mg L^{-1} hardness (as $CaCO_3$)			
Nominal U concentration (µg L^{-1})[a]	Measured U concentration (µg L^{-1})[a]	% Population growth	95% C.I.	Nominal U concentration (µg L^{-1})[a]	Measured U concentration (µg L^{-1})[a]	% Population growth	95% C.I.	Nominal U concentration (µg L^{-1})[a]	Measured U concentration (µg L^{-1})[a]	% Population growth	95% C.I.
0.1	0.11	100	0	0.1	0.11	100	0	0.1	0.11	100	0
5	6.8	96	6.6	25	21.0	97	5.4	25	21.7	100	0
10	10.3	88	10.0	50	50.0	96	4.7	50	42.2	92	9.2
30	31.9	90	12.8	75	68.2	99	1.3	75	62.1	90	6.0
50	61.6	83	9.4	100	92.5	89	6.1	100	87.4	81	9.8
70	69.2	82	11.1	130	126.9	86	9.5	125	118.6	84	7.5
100	98.0	73	9.6	150	149.9	87	9.3	150	130.0	79	9.9
110	102.6	57	6.6	170	161.9	58	10.1	175	182.4	70	8.7
125	118.0	43	12.7	180	183.0	56	12.1	200	186.6	59	7.8
140	125.4	47	6.3	200	199.9	33	13.9	225	206.9	52	13.3
150	161.9	18	20.2	225	228.0	5	5.4	235	259.9	50	9.6
175	183.5	5	2.0	250	242.1	2	2.7	245	273.6	36	6.6
200	213.2	1	1.7	300	315.8	0	0	250	234.8	11	7.7
400	421.8	0	0	400	419.5	0	0	300	282.7	11	7.4
				600	606.5	0	0	500	444.6	0	0
				1000	1039.7	0	0	750	828.8	0	0

[a] Uranium (U) concentration is expressed as uranyl (UO_2); this is derived by multiplying the U concentration by 1.14.

Table G.4 Percent survival response of *Mogurnda mogumda* to a range of U concentrations at three hardness levels.

Nominal U concentration ($\mu g\ L^{-1}$)[a]	6.6 mg L^{-1} hardness (as $CaCO_3$)			165 mg L^{-1} hardness (as $CaCO_3$)			330 mg L^{-1} hardness (as $CaCO_3$)		
	Measured U concentration ($\mu g\ L^{-1}$)[a]	% Survival	95% C.I.	Measured U concentration ($\mu g\ L^{-1}$)[a]	% Survival	95% C.I.	Measured U concentration ($\mu g\ L^{-1}$)[a]	% Survival	95% C.I.
First investigation									
0.1	0.11	98	3.3	0.11	98	3.3	0.11	95	6.7
500	434	98	3.3	458	98	3.3	514	100	0
1000	1128	98	3.3	1102	85	18.8	1046	87	12.0
1200	1208	100	0	1311	57	23.6	1277	48	17.1
1400	1448	95	6.7	1519	18	12.8	1453	25	13.1
1600	1528	65	15.0	1661	7	9.7	1573	8	10.6
1800	1944	45	13.1	1853	0	0	1910	18	22.9
2000	1972	13	9.7	2098	0	0	2291	0	0
Second investigation									
0.1	0.11	98	3.3	0.11	100	0	0.11	100	0
500	478	100	0	483	100	0	518	100	0
1000	1025	93	6.5	1045	97	4.1	1139	88	9.4
1200	1222	90	0	1239	90	0	1214	87	0
1400	1533	88	11.8	1511	65	21.9	1533	80	11.3
1600	1835	73	29.4	1773	48	29.3	1989	42	22.3
1800	1947	43	26.1	1900	32	23.4	2035	20	15.2
2000	2234	30	19.6	2242	15	18.8	2360	5	4.4

[a] Uranium (U) concentration is expressed as uranyl (UO_2); this is derived by multiplying the U concentration by 1.14.

Table G.5 Percent population growth response of *Hydra viridissima* to a range of Cu concentrations at two alkalinity levels.

Nominal Cu concentration ($\mu g\ L^{-1}$)	4 mg L^{-1} alkalinity (as $CaCO_3$)			102 mg L^{-1} alkalinity (as $CaCO_3$)		
	Measured Cu concentration ($\mu g\ L^{-1}$)	% Population growth	95% C.I.	Measured Cu concentration ($\mu g\ L^{-1}$)	% Population growth	95% C.I.
0.7	0.7	100	0	0.7	100	0
1	0.9	97	4.3			
2	1.7	86	5.7	1.8	84	7.0
3	2.4	78	10.3	2.4	76	6.0
4	3.0	66	4.4	3.4	63	9.2
5	4.0	54	10.8	5.3	57	2.8
6	5.2	48	5.8			
8	7.0	28	10.4	7.0	27	3.9
10	9.0	17	6.2			
12	10.0	10	5.9	10.0	4	4.1
15	14.0	0.5	0.9			
20	18.0	0	0	20.9	0	0

Table G.6 Percent population growth response of *Hydra viridissima* to a range of U concentrations at two alkalinity levels.

4 mg L^{-1} alkalinity (as $CaCO_3$)				102 mg L^{-1} alkalinity (as $CaCO_3$)			
Nominal U concentration (µg L^{-1})[a]	Measured U concentration (µg L^{-1})[a]	% Population growth	95% C.I.	Nominal U concentration (µg L^{-1})[a]	Measured U concentration (µg L^{-1})[a]	% Population growth	95% C.I.
0.1	0.11	100	0	0.1	0.11	100	0
25	21.0	97	5.4	20	19.3	90	14.6
50	50.0	96	4.7	50	57.8	86	9.1
75	68.2	99	1.3	75	81.6	81	12.9
100	92.5	89	6.1	100	109.6	72	22.4
130	126.9	86	9.5	125	130.3	70	11.5
150	149.9	87	9.3	150	171.4	53	10.45
170	161.9	58	10.1	170	194.6	40	15.9
186	183.0	56	12.1	200	225.2	12	7.8
200	199.9	33	13.9	250	303.2	0	0
225	228.0	5	5.4	400	517.6	0	0
250	242.1	2	2.7				
300	315.8	0	0				
400	419.5	0	0				
600	606.5	0	0				
1000	1039.7	0	0				

[a] Uranium (U) concentration is expressed as uranyl (UO_2); this is derived by multiplying the U concentration by 1.14.

Appendix H Summary data for metal speciation graphs

Table H.1 Predicted speciation (% distribution) of Cu in test water at pH 6.0 at three hardness levels.

Hardness (mg L^{-1}; as $CaCO_3$)	Cu concentration (µg L^{-1})	Cu species		
		% Cu^{2+}	% $CuOH^+$	% $CuCO_3$
6.6	0.7	95.8	3.2	0.33
	7	95.8	3.2	0.33
	70	95.8	3.2	0.33
165	0.7	96.6	2.8	0.27
	7	96.6	2.8	0.27
	70	96.6	2.8	0.27
330	0.7	96.8	2.6	0.24
	7	96.8	2.6	0.24
	70	96.8	2.6	0.24

Table H.2 Predicted speciation (% distribution) of U in test waters at pH 6.0 at three hardness levels. Uranyl species comprising < 2% total U were not included.

Hardness (mg L^{-1}; as $CaCO_3$)	U concentration ($\mu g\ L^{-1}$)	U species					
		% UO_2^{2+}	% UO_2OH^+	% $UO_2(OH)_2$	% $(UO_2)_3(OH)_5^+$	% UO_2CO_3	% $(UO_2)_2(OH)_3CO_3^-$
6.6	0.11	9.6	57.1	6.1	0	25.8	0.06
	10	9.1	54.4	5.9	0.07	24.6	4.6
	50	7.8	46.5	5.0	1.2	21.0	16.9
	75	7.3	43.1	4.6	2.0	19.5	21.8
	100	6.8	40.4	4.4	3.0	18.2	25.5
	150	6.1	36.2	3.9	4.9	16.3	30.7
	200	5.6	33.2	3.6	6.6	14.9	34.3
	400	4.3	25.7	2.8	12.4	11.5	41.0
	600	3.7	21.7	2.3	16.7	9.6	43.4
	800	3.2	19.0	2.1	20.0	8.4	44.4
	1000	2.9	17.1	1.8	22.8	7.5	44.7
165	0.11	11.1	57.4	5.9	0	24.4	0.05
	10	10.6	54.8	5.6	0.07	23.2	4.4
	50	9.1	47.1	4.8	1.1	20.0	16.3
	75	8.5	43.8	4.5	2.0	18.5	21.1
	100	7.9	41.1	4.2	2.9	17.4	24.7
	150	7.1	36.9	3.8	4.7	15.6	30.0
	200	6.5	33.8	3.5	6.4	14.3	33.5
	400	5.1	26.3	2.7	12.1	11.0	40.3
	600	4.3	22.2	2.3	16.4	9.3	42.8
	800	3.8	19.5	2.0	19.7	8.1	43.8
	1000	3.4	17.6	1.8	22.5	7.3	44.2

Table H.2 Cont'd

Hardness (mg L^{-1}; as $CaCO_3$)	U concentration ($\mu g\ L^{-1}$)	U species					
		% UO_2^{2+}	% UO_2OH^+	% $UO_2(OH)_2$	% $(UO_2)_3(OH)_5^+$	% UO_2CO_3	% $(UO_2)_2(OH)_3CO_3^-$
330	0.11	11.9	57.5	5.8	0	23.6	0.05
	10	11.4	54.9	5.5	0.07	22.5	4.3
	50	9.8	47.4	4.8	1.1	19.4	16.0
	75	9.1	44.1	4.4	1.9	18.0	20.7
	100	8.6	41.9	4.2	2.8	16.9	24.3
	150	7.7	37.3	3.7	4.6	15.2	29.5
	200	7.1	34.1	3.4	6.3	13.9	33.0
	400	5.5	26.6	2.7	12.0	10.8	39.9
	600	4.7	22.5	2.3	16.2	9.1	42.4
	800	4.1	19.8	2.0	19.6	7.9	43.5
	1000	3.7	17.8	1.8	22.3	7.1	43.9

[a] Uranium (U) concentration is expressed as uranyl (UO_2); this is derived by multiplying the U concentration by 1.14.

Table H.3 Predicted speciation (% distribution) of Cu in test waters at pH 6.0 at two alkalinity levels.

Alkalinity (mg L^{-1}; as $CaCO_3$)	Cu concentration (µg L^{-1})	Cu species		
		% Cu^{2+}	% $CuOH^+$	% $CuCO_3$
4	0.7	95.8	3.2	0.33
	7	95.8	3.2	0.33
	70	95.8	3.2	0.33
102	0.7	89.9	2.5	0.33
	7	89.9	2.5	0.33
	70	89.9	2.5	0.33

Table H.4 Predicted speciation (% distribution) of U in test waters at pH 6.0 at two alkalinity levels. Uranyl species comprising < 2% total U were not included.

Alkalinity (mg L^{-1}; as $CaCO_3$)	U concentration (µg L^{-1})	U species				
		% UO_2^{2+}	% UO_2OH^+	% UO_2CO_3	% $(UO_2)_2(OH)_3CO_3^-$	% $UO_2(CO_3)_2^{2-}$
4	0.11	9.6	57.1	25.8	0.06	
	10	9.1	54.4	24.6	4.6	
	50	7.8	46.5	21.0	16.9	
	75	7.3	43.1	19.5	21.8	
	100	6.8	40.4	18.2	25.5	
	150	6.1	36.2	16.3	30.7	
	200	5.6	33.1	14.9	34.3	
	400	4.3	25.7	11.5	41.0	
	600	3.7	21.7	9.6	43.4	
	800	3.2	19.0	8.4	44.4	
	1000	2.9	17.1	7.5	44.7	
102	0.11	1.3	6.9	67.3	0.02	23.5
	10	1.3	6.8	66.2	2.2	23.2
	50	1.3	6.4	62.6	7.0	21.9
	75	1.2	6.1	60.8	9.6	21.2
	100	1.2	6.0	58.6	12.3	20.6
	150	1.1	5.7	55.6	17.1	19.7
	200	1.1	5.5	53.6	20.3	18.7
	400	0.93	4.8	46.6	30.7	16.3
	600	0.84	4.3	42.0	37.4	14.7
	800	0.77	4.0	38.6	42.3	13.5
	1000	0.72	3.7	36.1	46.1	12.6

[a] Uranium (U) concentration is expressed as uranyl (UO_2); this is derived by multiplying the U concentration by 1.14.