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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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Summary

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 are, however, 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 research 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 $CaCO_3$ L^{-1}), at a constant pH (6.0), 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*, but had no effect on U toxicity. The opposite was observed for *H. viridissima*, where increased hardness had no effect on Cu toxicity, but it 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*, but 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.

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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

Nadine Riethmuller, Scott Markich, David Parry and Rick van Dam

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 mobilisation from sediments (eg Al and Fe). Both marine and freshwater ecosystems are threatened, however, soft freshwaters are particularly sensitive as they are poorly buffered and prone to acidification (McDonald & 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 & ARMCANZ 2001) to protect aquatic ecosystems.

The metals of greatest concern to tropical Australian freshwaters are Al, Cd, Co, Cu, Ni, Mn, Pb, U, V and Zn – largely as a result of mining activities, but also from urban impacts (refer to review by Markich & 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 (see Riethmuller (2000), 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 predominantly on toxicity data for Northern Hemisphere species (ANZECC & ARMCANZ 2001). 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 & Firth 1983, Markich & 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, that are beyond ranges so far studied in the Northern Hemisphere (Skidmore & 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 & Firth 1983). The inability of the previous 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 40% of the Australian continent (ASTEC 1993).

1.1.2 Water quality guidelines relevant to tropical Australia

The main objective of the Australian and New Zealand WQGs is to provide a guide for setting water quality objectives required to sustain current or likely future environmental values for natural and semi-natural water resources (ANZECC & ARMCANZ 2001). 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 & 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 on aquatic organisms inhabiting the Wet/Dry tropics. An interim U guideline for Australian freshwaters is 0.5 $\mu g\ L^{-1}$ (ANZECC & ARMCANZ 2001). However, there is no provision in the guidelines to use an algorithm to modify the guideline value to account for varying water hardness.

A recent review of available data on metal toxicity to aquatic biota in tropical Australia (Markich & 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 2000). 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 2000). 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 2000). 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 & Jeffree 1994).

The Australian and New Zealand water quality guidelines (ANZECC & ARMCANZ 2001) recognises 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 & Firth 1983, Nor 1987). The revised Australian and New Zealand water quality guidelines for the protection of aquatic ecosystems recommend a Cu concentration between 0.4 µg Cu L^{-1} at 6 mg $CaCO_3$ L^{-1} and 13 µg L^{-1} at 400 mg $CaCO_3$ L^{-1}, depending on the water hardness (ANZECC & ARMCANZ 2001). The hardness-dependent algorithm used is:

$$\mathrm{HMGV} = 1.4\left(\frac{\mathrm{H}}{30}\right)^{0.85}$$

where HMGV is the hardness modified guideline value (µg L^{-1}) and H is the measured hardness (mg $CaCO_3$ L^{-1}) of a fresh surface water.

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) (Leckie & Davis 1979, Flemming & 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 2000). 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 2000).

Table 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 & 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 & 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 2000). 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 & 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 & 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 & Davis 1979, Dzombak & 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 & Morel 1990). The majority of Cu^{2+} is sorbed around pH 7 in most fresh surface waters (Moore & Ramamoorthy 1984, Dzombak & 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 (see Riethmuller (2000), Appendix A), inhabiting tropical Australian freshwaters has been determined. A recent review by Markich and Camilleri (1997) details this information. Only those studies, that 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 is difficult to compare (table 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 $CaCO_3$ L^{-1}). 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 & 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 $CaCO_3$ L^{-1}, 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 & Holdway 1999). Allison and Holdway (1988) also reported *H. viridissima* to be a more sensitive species than *H. vulgaris* to U (table 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 with that causing 50% mortality, also has important behavioural implications in assessing environmental impacts.

Table 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]	24 h population growth 48 h population growth 48 h feeding inhibition 96 h population growth	410 (LC_{50})[g] 190 (LC_{50})[g] 10 (EC_{50})[d] 40 (LC_{50})[g]	Beach & Pascoe (1998)
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)

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.

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

c MDEC, minimal detectable effect concentration (Ahsanullah & 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

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 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 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 water (Buffalo Billabong), 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 with those investigated in independent studies (see Riethmuller (2000), Appendix A). This supports the findings of Bywater et al (1991) for U sensitivity.

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 1995). 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 & Holdway 1999). Copper has been found to inhibit the photosynthesis of an Australian tropical *Chlorella* sp at 1.6 µg L^{-1} Cu (Franklin et al 1998). At non-toxic concentrations, Cu has been reported to increase hydra population growth (Stebbing & Pomroy 1978, Pollino & Holdway 1999). Hormesis was found to occur at Cu concentrations below 5 µg L^{-1} for *H. littoralis* (Stebbing & Pomroy 1978) and below 8 µg L^{-1} for *H. vulgaris* and *H. viridissima* (Pollino & Holdway 1999).

Gill surfaces of fish have been identified as the primary uptake site of several waterborne metals (Cu^{2+}, Laurén & McDonald 1986, Reid & McDonald 1991, Cd^{2+}, Part et al 1985, Reid & 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 & 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 & McDonald 1991). These mechanisms can be used to describe osmoregulation inhibition by Cu^{2+} exposure (Laurén & McDonald 1986, Reid & McDonald 1988). Copper(II) has been found to disrupt gill functioning by forming covalent bonds with nitrogen/sulphur-rich ligands such as those of APTase (Reid & McDonald 1988).

Surface bound Ca^{2+} is known to stabilise the gill membrane, consequently reducing ionic permeability (Flik & Verbost 1994). It is hypothesised 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 & 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 & 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 ligands) and binding affinity (Reid & 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 (see 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 & Sprague 1978, Gauss et al 1985, Belanger et al 1989) that 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 µg L^{-1} in soft acid water, to 520 µg L^{-1} in hard alkaline water, where hardness ranged from 30 to 360 mg $CaCO_3$ L^{-1}, 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 & Mackay 1980, Mierle 1981, Horne & 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 affecting Cu speciation (Markich & 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 & 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 & Mackay 1980, Laurén & 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 & Stokes 1985, Cusimano et al 1986, Macfie et al 1994, Horne & 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 & 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 & 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); other studies suggest that under certain conditions Cu toxicity may be enhanced (Guy & 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) are 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 & 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 µg L^{-1} U (Hart et al 1987, Markich 1998). Uranium is non-essential for biological processes and is generally toxic at elevated concentrations (Berlin & Rudell 1979). Since U is highly soluble and mobile in natural waters (Morse & 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 & 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 oxidised surface waters and forms stable, readily soluble, cationic, anionic and/or neutral complexes that are highly mobile (Langmuir 1978, Osmond & 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-dependent. 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 et al (2000) provided evidence to suggest that UO_2^{2+} and, to a

lesser extent, UO_2OH^+ are the U species that 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 $CaCO_3$ L^{-1}) 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 with uranyl complexes with carbonate and phosphate in freshwaters (Gascoyne 1992). Uranyl-phosphate complexes only start to become significant when the concentration of phosphate approaches 100 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 that 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 2000). 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 (see Riethmuller (2000), 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 that 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 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 5-fold more sensitive to U than the pink hydra, *H. vulgaris* (table 3). Investigations using natural Magela Creek (Buffalo Billabong) water found U concentrations ≥160 µg L^{-1} inhibit *H. viridissima* population growth (Allison & Holdway 1988). However, in synthetic Magela Creek water, *H. viridissima* was three times more sensitive to U with an EC_{50} of 108 µg L^{-1} (Markich & 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.

Table 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 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 (LC_{50})[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 3 Cont'd

Species	Water type	pH	Hardness (mg CaCO3 L-1)	Alkalinity (mg CaCO3 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 UO2); this was derived by multiplying the U concentration by 1.14.
b BEC_{10}, 10% bounded-effect concentration (Hoekstra & van Ewijk 1993), an analogous statistical measure of the no-observed effect concentration (NOEC)
c MDEC, minimal detectable concentration (Ahsanullah & 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

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.

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 & 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 that 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 & 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 principal 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 & 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 $CaCO_3$ L^{-1} hardness, 53 mg $CaCO_3$ L^{-1} alkalinity, pH 7.5) to be approximately 4-fold greater than in soft water (35 mg $CaCO_3$ L^{-1} hardness, 11 mg $CaCO_3$ L^{-1} alkalinity, 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 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 2000). For example, Markich et al (2000) found a decrease in pH from 6.0 to 5.0 to have a 5-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 2000), Franklin et al (1998) observed that a decrease in pH from 6.5 to 5.7 had a 2-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 & 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 & 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* (see Riethmuller (2000), 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 was assessed using existing protocols from the Environmental Research Institute of the Supervising Scientist (Hyne et al 1996, Markich & Camilleri 1997). The protocols are detailed in Appendix B of Riethmuller (2000). 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 µS 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) (fig 1). This water is very soft (2–4 mg $CaCO_3$ L^{-1}), 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.

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 $UO_2SO_4.3H_2O$ respectively in high purity deionised water (Milli-Q). The stock solutions were prepared in pre-cleaned 1 L 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.

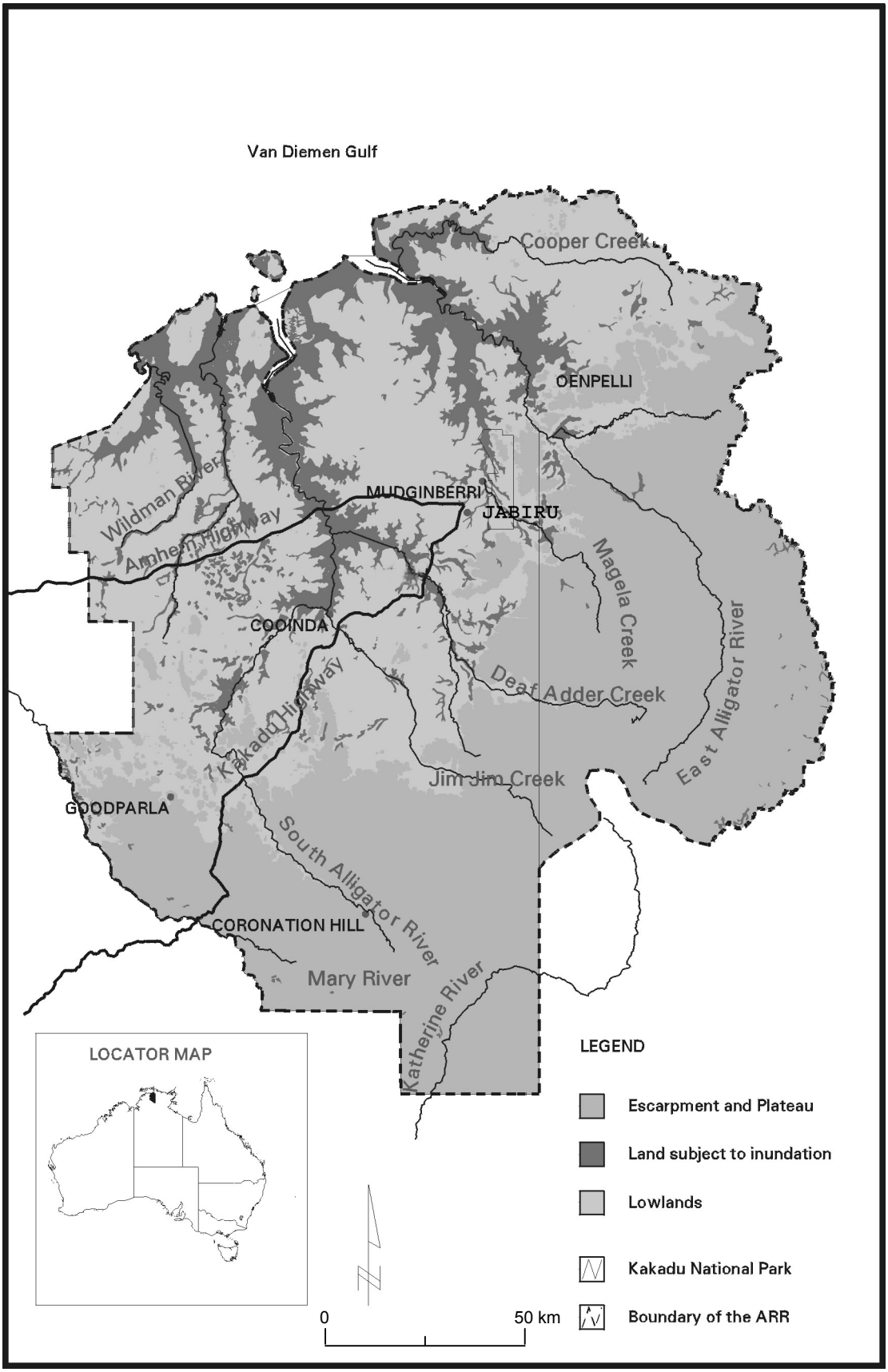

Figure 1 Map of the Alligator Rivers Region, Northern Territory, including the Magela Creek catchment

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

2.2.1 Bioassay selection

Freshwater cnidaria have several inherent advantages for use in bioassays:

- Hydra are diploblastic (ie has only two epithelial layers), so all cells are in contact with the surrounding medium (Beach & Pascoe 1998);
- Hydra reproduce asexually, so all the animals in the stock culture are genetically very similar (Beach & Pascoe 1998);
- Low genetic variation among test individuals minimises experimental variation due to differences in morphology, and maximises experimental reproducibility;
- Hydra are able to withstand extensive manipulation, and are easily reared and maintained in the laboratory (Blaise & Kusui 1997);
- Hydra assays are simple, cost effective and easy to conduct (Blaise & 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* (see Riethmuller (2000), Appendix A).

2.2.3 Stock culture maintenance

Hydra viridissima were originally collected from Magela Creek (fig 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 Riethmuller (2000) 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 analysed. The test was considered valid if control population growth was greater than 20 individuals after 96 h. Riethmuller (2000) Appendix B describes the protocol in more detail.

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

2.3.1 Bioassay selection

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

- Fish communities represent various trophic classes and use foods from aquatic and terrestrial sources, providing an integrative view of the watershed;
- Both acute toxicity (fish mortality) and stress effects (depressed growth or reproductive success) can be evaluated;
- 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;
- Being relatively long-lived, fish provide temporal integration in assessments.

The present study used *M. mogurnda* sac-fry, because 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 the purple-spotted or northern trout gudgeon (Merrick & Schmida 1984). The freshwater species has a wide distribution throughout northern Australia, extending south to Lake Eyre and north-east coast regions (Merrick & Schmida 1984). *Mogurnda mogurnda* are carnivorous and the sexes are dimorphic (Merrick & 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. Riethmuller (2000) 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, the eggs 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. Riethmuller (2000) 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 (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 (4 mL) 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 oxidised states. In oxidised waters it is the hexavalent state (UO_2^{2+}, uranyl ion) that predominates and therefore has been used to represent U in this study (Markich & 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 and Brown (1999). 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 (see section 3.2.3). Population growth and survival were measured as a percentage of control, where the control response equalled 100%. Data derived for each hardness or alkalinity level were pooled and a mean and 95% confidence interval (CI) 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% CI were 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 ($\alpha = 0.05$). 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 & 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 & ARMCANZ 2001). 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 & 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 & 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 section 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 Water Resources Branch of the Department of Lands, Planning and Environment and the Environmental Research Institute of the Supervising Scientist to determine a relevant range of hardness for tropical Australian freshwater systems (fig 2, table 4).

Three levels of hardness were selected – 6.6, 165 and 330 mg $CaCO_3$ L^{-1}. The baseline hardness of 6.6 mg $CaCO_3$ L^{-1} represents the hardness of Magela Creek water (fig 2, table 4). The rationale for using Magela Creek water as a baseline reference in this study is outlined in Riethmuller (2000) 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 (fig 2, table 4).

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 $Mg(NO_3)_2$. The population growth of *H. viridissima* and *M. mogurnda* sac-fry survival in treatments with 165 and 330 mg $CaCO_3$ L^{-1} hardness were compared with growth in 6.6 mg $CaCO_3$ L^{-1} hardness, using the protocols described in Riethmuller (2000) Appendix B. In synthetic water at 6.6 mg $CaCO_3$ 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 (see Riethmuller (2000), Appendix B.1.13 & 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 5).

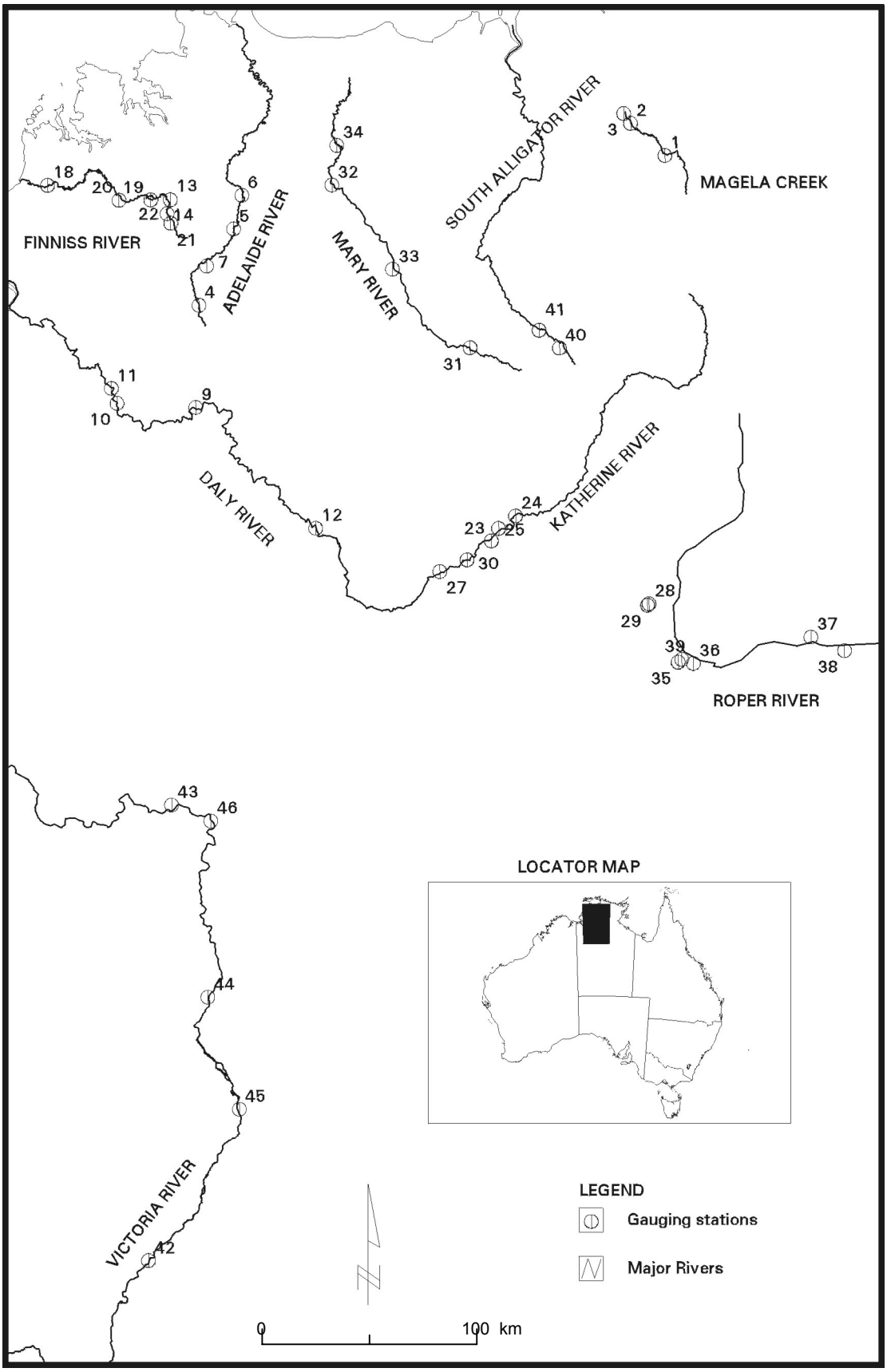

Figure 2 Location of gauging stations along several Northern Territory water systems. Gauging station numbers correspond to table 4.

Table 4 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 2.

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***)	6.0	5.7	3.7
3	Magela Ck at Mudginberri Billabong	6.2	5.4	4.3
4	Adelaide River 8 km downstream of Daly Road	6.9		45
5	Adelaide River at Tortilla Flats	7.3	92	93
6	Adelaide River upstream Marrakai Crossing	6.8	83	93
7	Adelaide River at Railway Bridge	7.1	75	55
9	Daly River at Beeboom Crossing 2 km downstream	8.3		260
10	Daly River at Mount Nancar	7.8	18	150
11	Daly River at Police Station	8.2		200
12	Daly River at upstream Dorisville Crossing	7.8	220	210
13	East Finniss River at Rum Jungle	5.9	69	8.7
14	Finniss River at Batchelor Damsite	7.1	110	140
18	Finniss River at Point 1	6.2		89
19	Finniss River at Point 10	6.9	58	50
20	Finniss River at Point F (Bad Crossing)	5.8	5.5	14
21	Finniss River at Point L	7.2	58	90
22	Finniss River at Taw 2	6.4		120
23	Katherine River at Donkey Camp Outflow	7.1	9.5	12
24	Katherine River at Gorge Caravan Park	6.1		11
25	Katherine River at inflow to Donkey Camp	6.3	7	9
27	Katherine River at PT'Am'	7.8	370	300
28	Katherine River Site 43	7.9	280	280
29	Katherine River Site 44	7.9	290	280
30	Katherine River downstream Sewage Ponds Outflow	6.9		170
31	Mary River at El Sherana Rd Crossing	6.6		20
32	Mary River at Mount Bundey	6.5		26
33	Mary River at Pt.9a	6.7		22
34	Rockhole Weedsite at Corroboree – Mary River	5.8	15	12
35	Roper River – Mataranka Homestead Crossing	6.5	36	330
36	Roper River at downstream Mataranka Homestead	7.8	480	270
37	Roper River at downstream Moraok Homestead	8.2		280
38	Roper River at Mole Hill	7.3	252	200
39	Roper River at Thermal Springs Mataranka	7.1	480	448
40	South Alligator River at Coronation Hill	7.1	50	52

Table 4 Cont'd

Station no.	Station name	pH	Hardness (mg $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})
41	South Alligator River at El Sharana ('c')	7.7	53	36
42	Upper Victoria R. at Wave Hill Police Station	7.7	210	170
43	Victoria River at Coolibah Homestead	8.2	220	210
44	Victoria River at Dashwood Crossing	8.0	260	190
45	Victoria River at Pigeon Hole Homestead	7.8	1.8	150
46	Victoria River at Victoria Highway	8.1	1.9	210

Table 5 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 $CaCO_3$ L^{-1}). The number of organisms at 0 h equalled 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 Riethmuller (2000) 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 $CaCO_3$ L^{-1}) and pH (6.0 ± 0.3). Raw data for each test-series of a given metal-organism exposure are provided in Riethmuller (2000) 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 and 4, respectively. Summary data for each concentration-response curve are given in Riethmuller (2000) Appendix G, tables 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 $CaCO_3$ L^{-1}), are given in table 6.

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 6). 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 6). 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 6).

The BEC_{10}, MDEC, NOEC and LOEC values are consistent with the trend reflected by the EC_{50} values (table 6), 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 6). 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 6). However, a two-fold increase in water hardness (ie from 165 to 330 mg $CaCO_3$ L^{-1}) did not significantly ($P > 0.05$) affect Cu toxicity (ie overlapping 95% confidence intervals of the LC_{50} values, table 6).

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 6). 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 6).

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 $CaCO_3$ L^{-1}) is given in figure 5. 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%, see Riethmuller (2000), Appendix H).

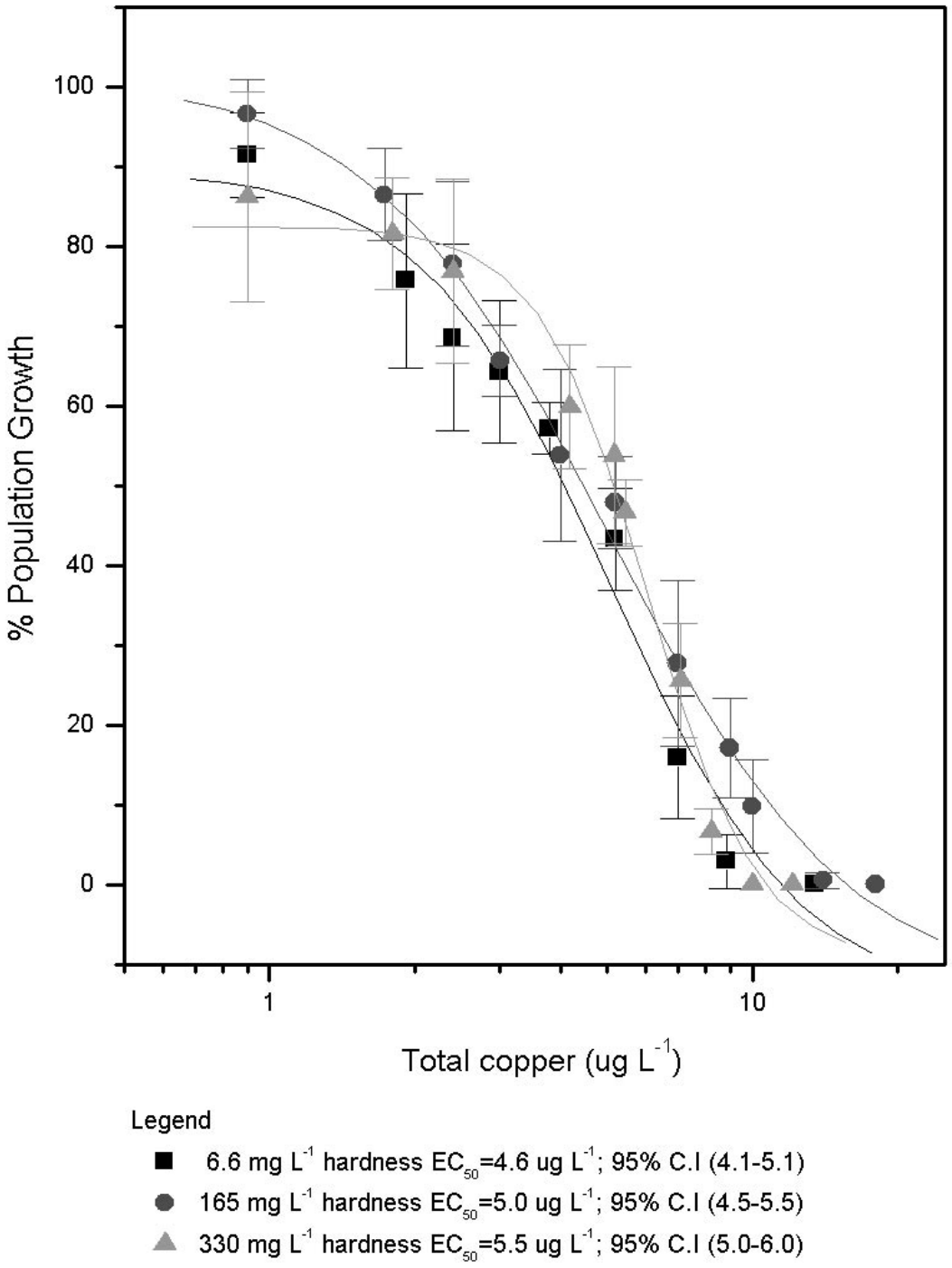

Figure 3 Population growth of *H. viridissima* exposed to Cu over 96 h at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or nine replicates ± 95% CI.

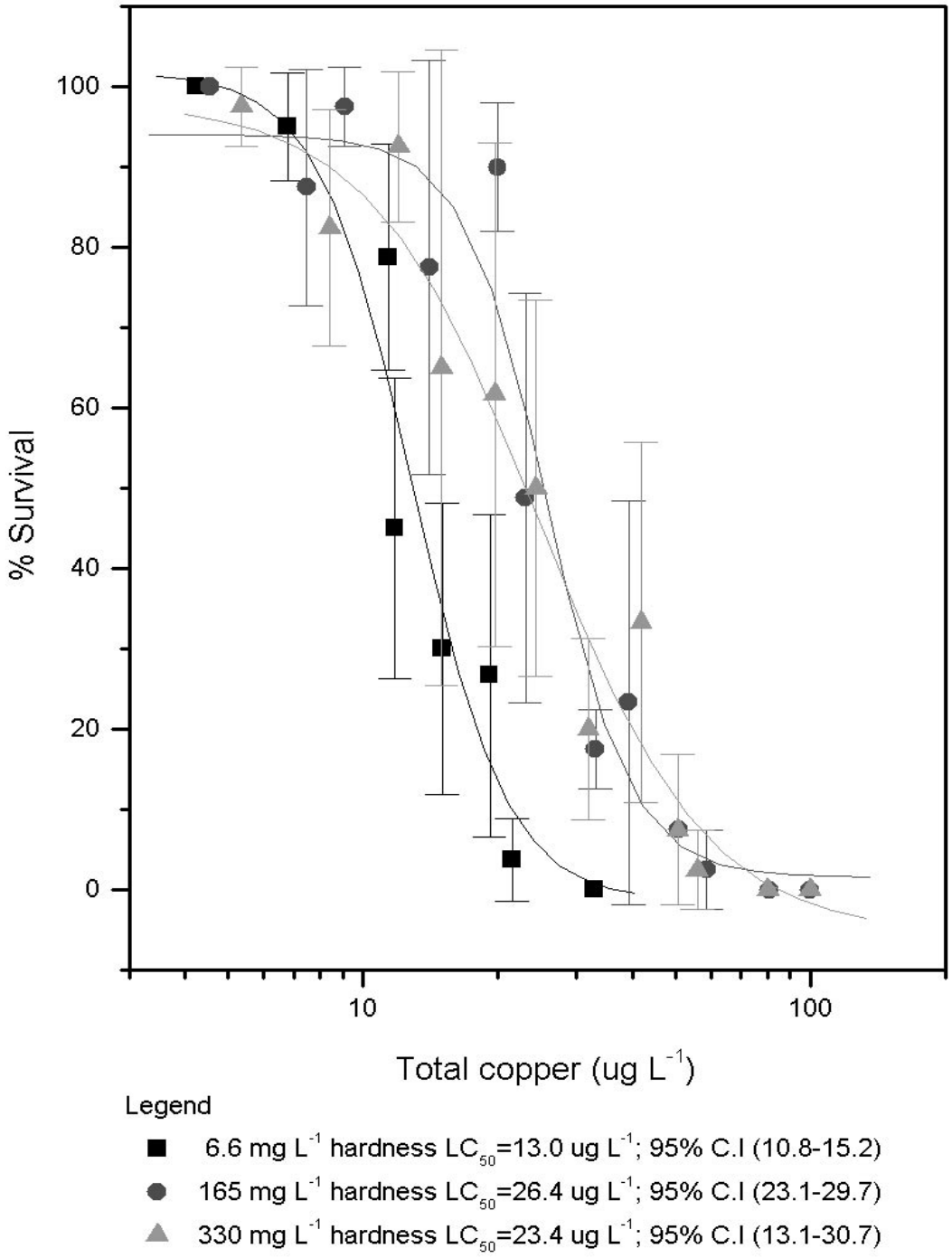

Figure 4 Survival of *M. mogurnda* exposed to Cu over 96 h at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or eight replicates ± 95% CI.

Table 6 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 $CaCO_3$ L^{-1}) and pH (6.0 ± 0.3) conditions, for 96 h

Species	Hardness (mg $CaCO_3$ L^{-1})	BEC_{10}	MDEC	NOEC	LOEC	Effect concentration (95% CI)
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})

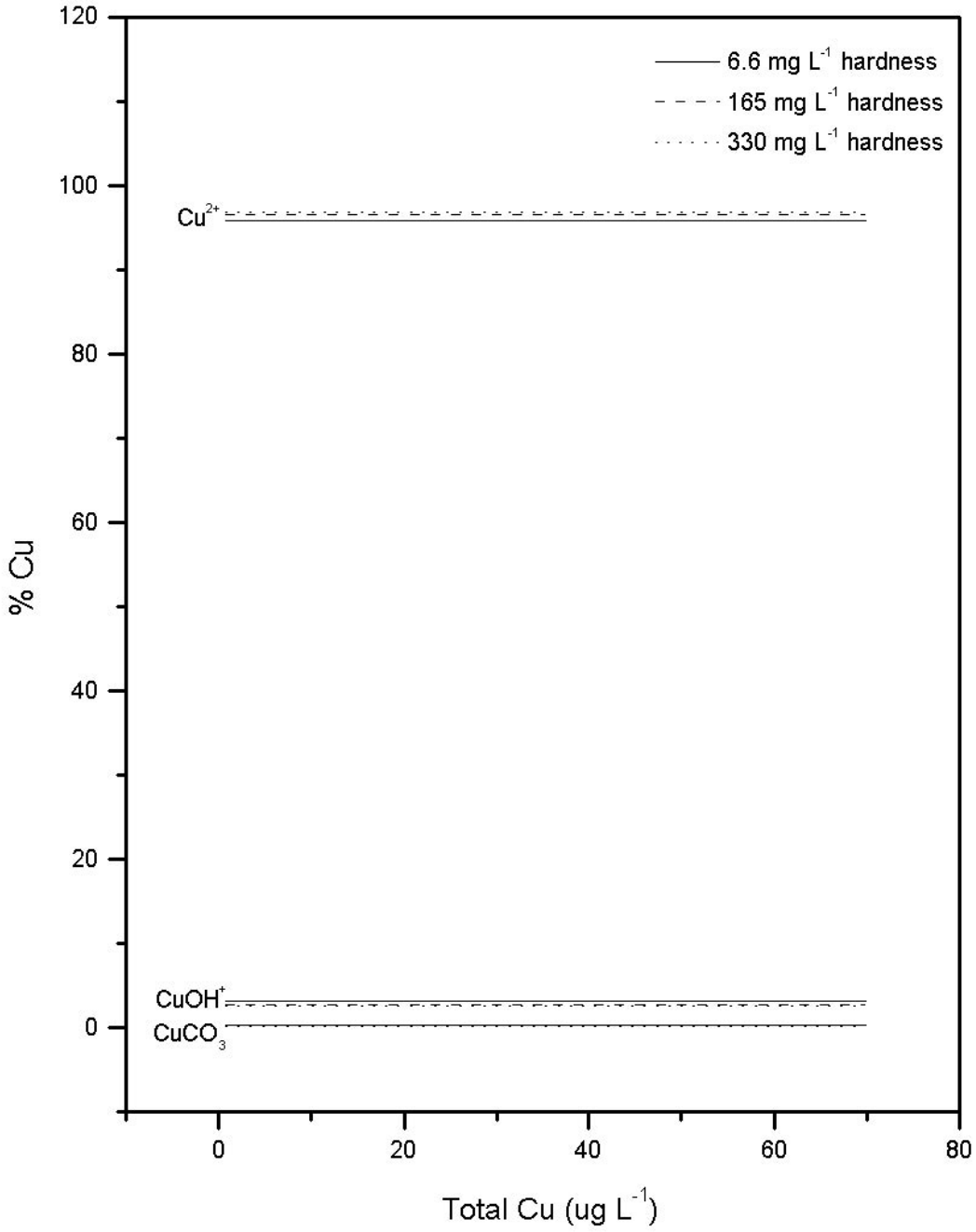

Figure 5 Predicted speciation (% distribution) of Cu in test water (pH 6.0) at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1})

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 6. 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 7 and 8, respectively. Summary data for each concentration-response curve is given in Riethmuller (2000) 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 $CaCO_3$ L^{-1}) are given in table 7.

H. viridissima

Based on the EC_{50} values, a 25-fold increase in water hardness (ie from 6.6 to 165 mg $CaCO_3$ L^{-1}) 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 7). A 50-fold increase in water hardness (ie from 6.6 to 330 mg $CaCO_3$ L^{-1}) 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 7). A 2-fold increase in water hardness (ie from 165 to 330 mg $CaCO_3$ L^{-1}) 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 7).

The trend observed for the EC_{50} is not consistent with BEC_{10} and MDEC values given in table 7, due to differences between slopes of the concentration-response curves (fig 6). 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 7). 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 7). However, there was no difference in toxicity at the two higher hardness levels (ie overlapping 95% confidence intervals of the LC_{50} values, table 7). 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 7).

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 7), due to the steeper slope of the former concentration-response curve (fig 7). 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 7).

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 with that of the LC_{50} values (table 7). The lack of consistency can be attributed to differences between slopes of the concentration-response curves (fig 8). 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.

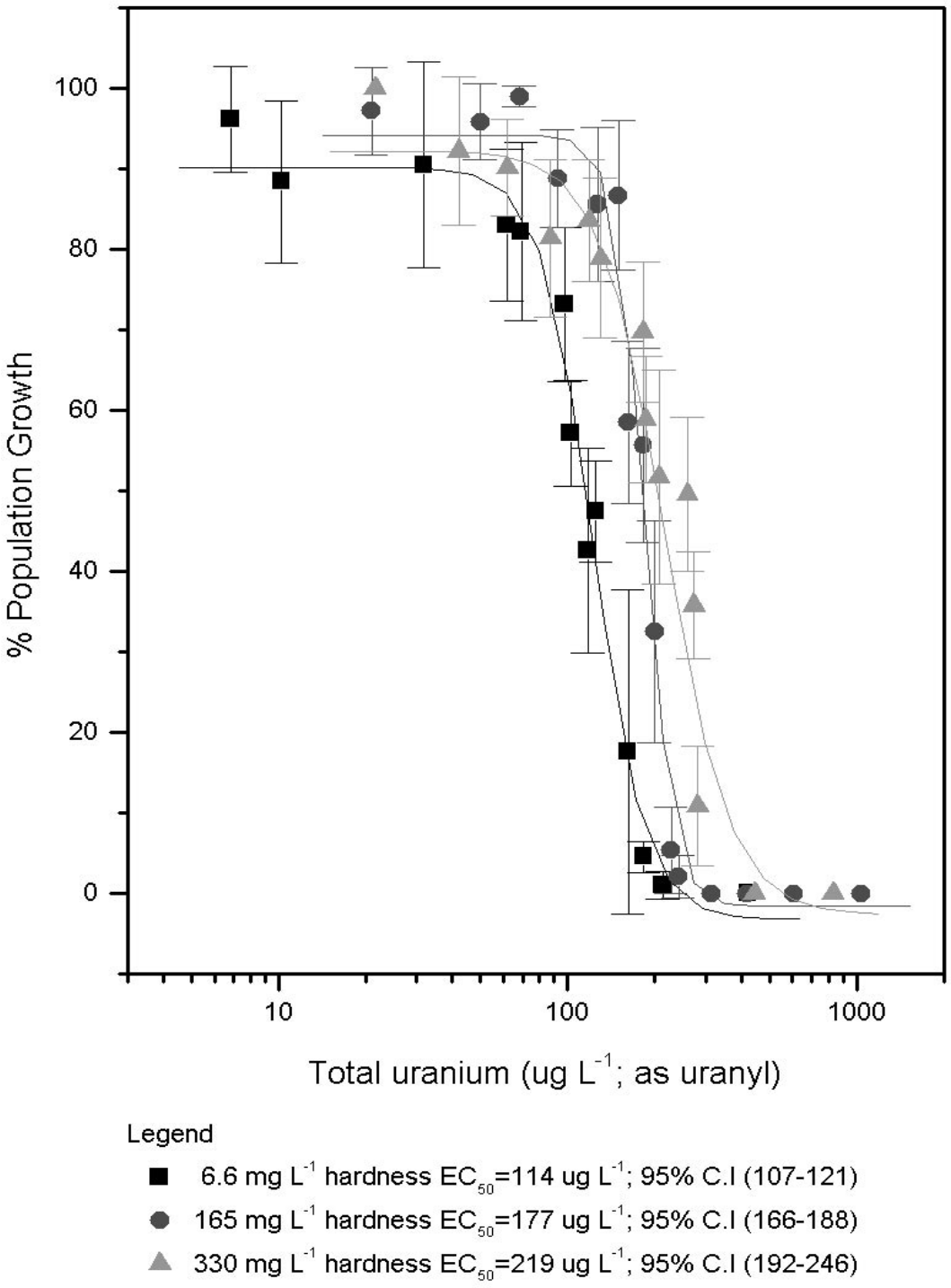

Figure 6 Population growth of *H. viridissima* exposed to U over 96 h at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or nine replicates ± 95% CI.

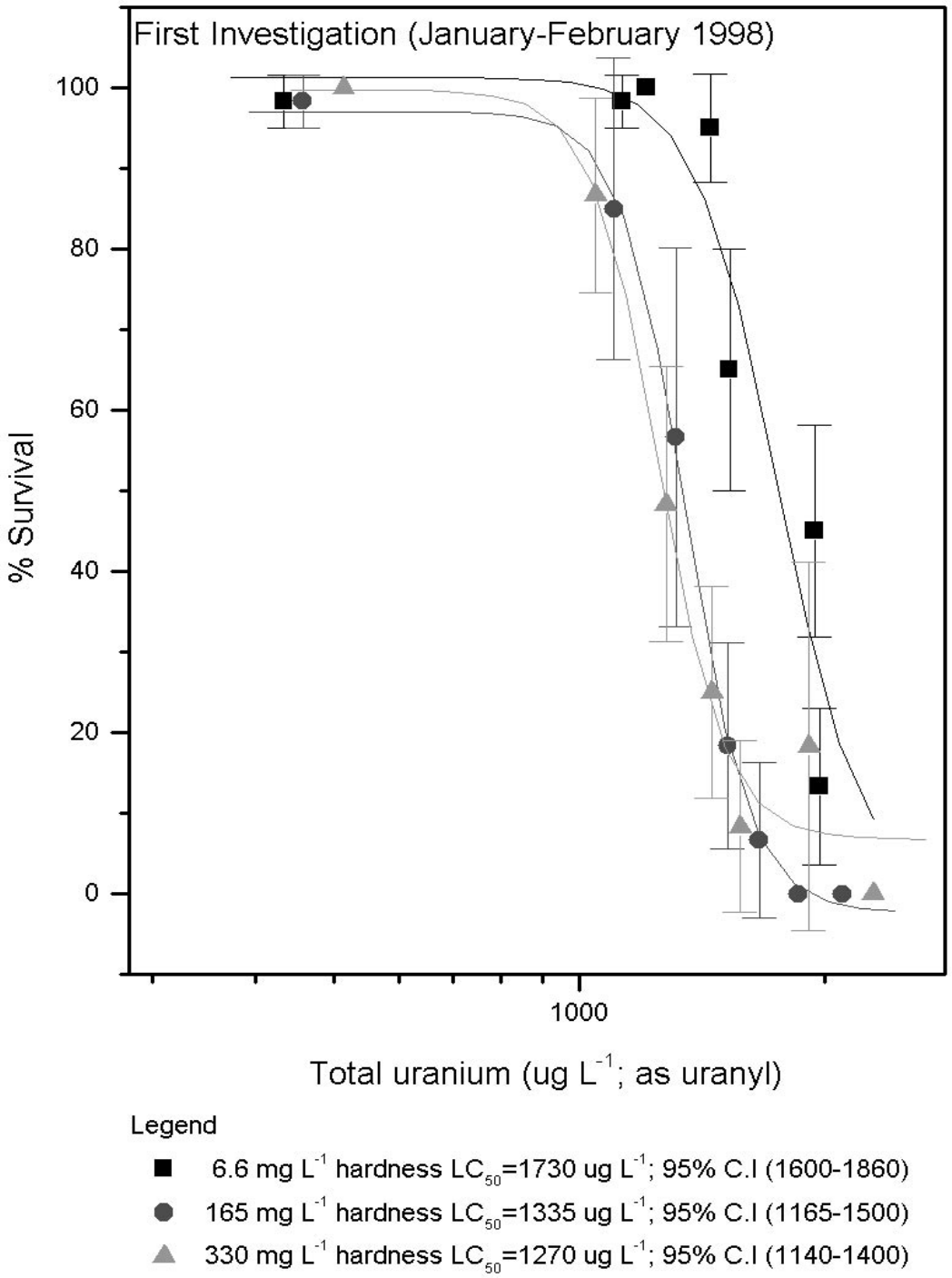

Figure 7 Survival of *M. mogurnda* exposed to U over 96 h at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or eight replicates ± 95% CI.

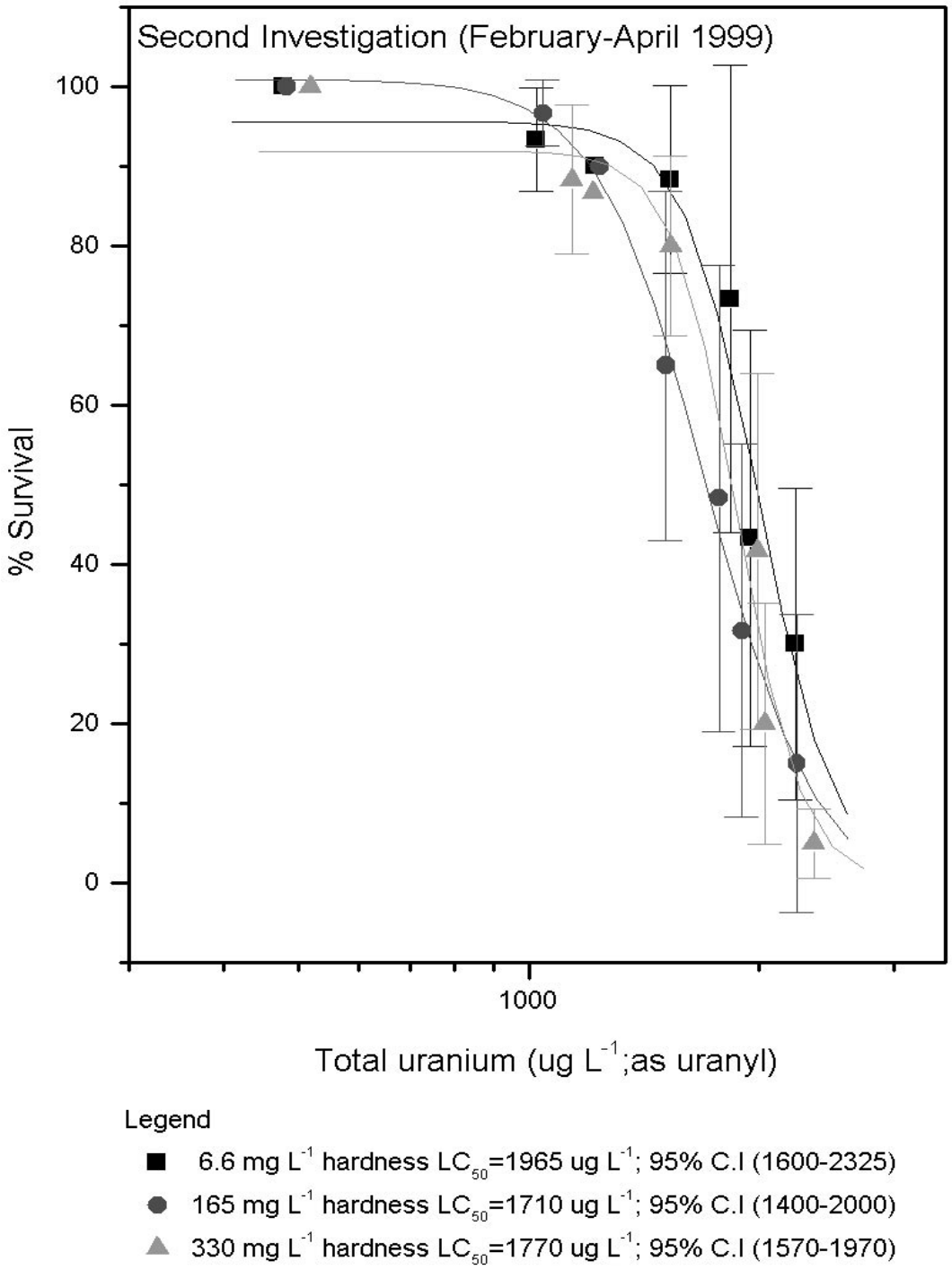

Figure 8 Survival of *M. mogurnda* exposed to U over 96 h at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or eight replicates ± 95% CI.

Table 7 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 $CaCO_3$ L^{-1})	BEC_{10}	MDEC	NOEC	LOEC	Effect concentration (95% CI)
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})

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 $CaCO_3$ L^{-1}) is given in figure 9. 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%, see Riethmuller (2000), 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%, see Riethmuller (2000), Appendix H).

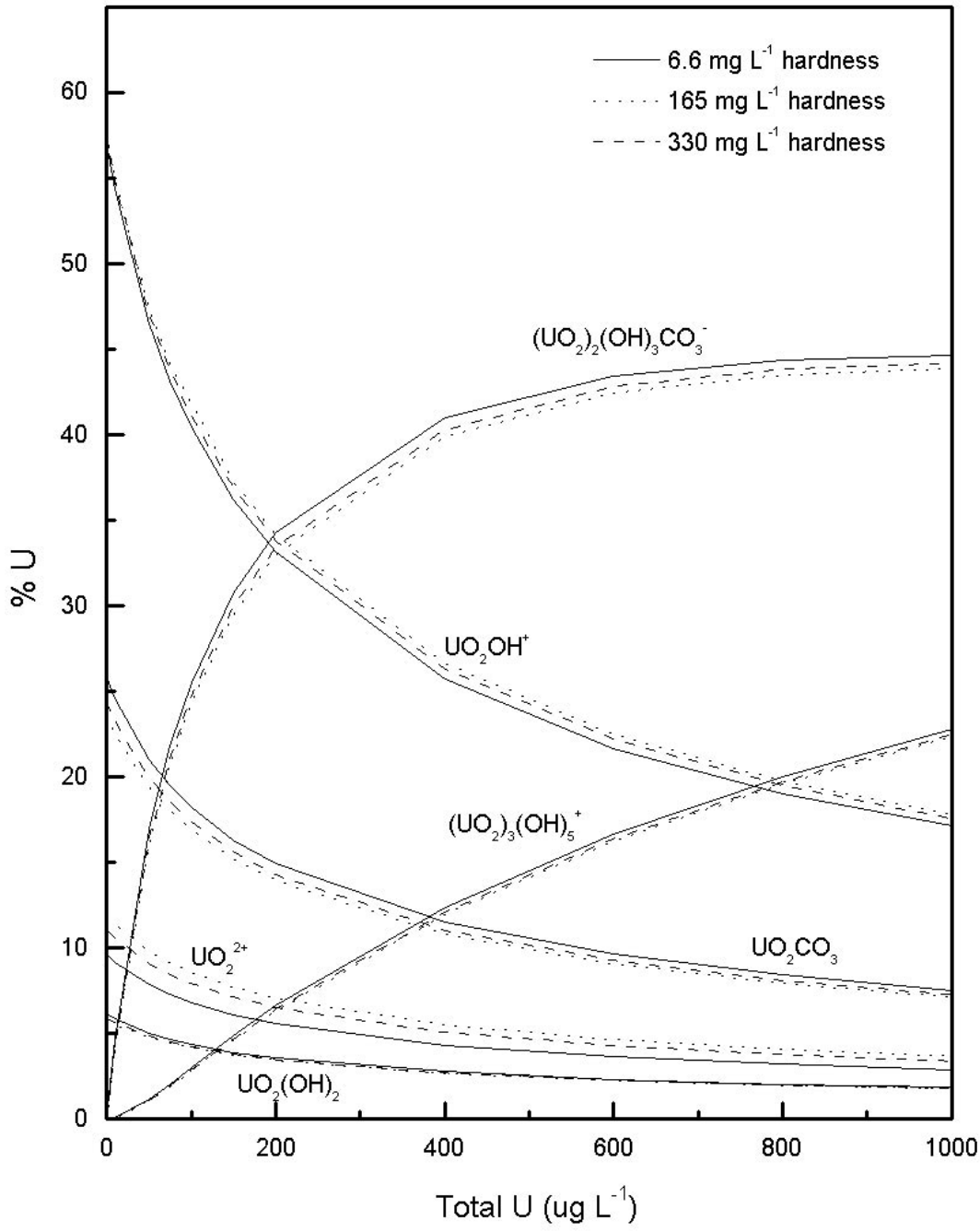

Figure 9 Predicted speciation (% distribution) of U in test water (pH 6.0) at three hardness levels (6.6, 165 and 330 mg $CaCO_3$ L^{-1}). Uranyl species comprising <2% total U are excluded for clarity.

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 with 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 with Markich and Camilleri's (1997) findings. The LC_{50} for *M. mogurnda* was 13.0 ± 2.2 µg L^{-1} in this study, compared with 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 more toxic to *M. mogurnda* at 6.6 than at 330 mg $CaCO_3$ 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 & 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 & 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 & Jeffree 1994). For example, Part et al (1985) reported 4 to 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 $CaCO_3$ L^{-1}) 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, that 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 $CaCO_3$ 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 $CaCO_3$ L^{-1} hardness, the sensitivity of *M. mogurnda* to U was found to be significantly ($P \leq 0.05$) less in this study compared with 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 with 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 & Jeffree 1994). Previous studies (Markich & 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 $CaCO_3$ L^{-1} than at 165 mg $CaCO_3$ 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 & 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 & 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 & 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 & Mackay 1980, Laurén & 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 (see section 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 the Water Resources Branch of the Department of Lands, Planning and Environment and the Environmental Research Institute of the Supervising Scientist to determine a relevant range of alkalinity for tropical Australian freshwater systems (fig 2, table 4). Three levels of alkalinity were selected – 4.0, 102 and 205 mg $CaCO_3$ L^{-1}. The baseline alkalinity of 4 mg $CaCO_3$ L^{-1} represents the mean alkalinity of Magela Creek water (see fig 2). The rationale for using Magela Creek water as a baseline reference in this study is outlined in Riethmuller (2000) Appendix B.1.4. The other alkalinity levels were calculated to compliment the hardness levels studied (ie 165 and 330 mg $CaCO_3$ L^{-1}) and represent tropical Australian waters (see fig 2, table 4).

4.2.2 Isolating alkalinity effects

Alkalinity was manipulated 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 precipitates. A white precipitate formed at an alkalinity of 205 mg $CaCO_3$ L^{-1} after 72 h, while the other solutions appeared free of precipitates.

A preliminary test was conducted to ensure the pH, conductivity, dissolved oxygen (DO) 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 & Mogan 1981). A few techniques were examined to stabilise pH, and these are detailed in Riethmuller (2000) 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 with growth measured in non-buffered treatments. The 5 mM buffer maintained the pH of 4 and 102 mg $CaCO_3$ L^{-1} alkalinity solutions within 6.0 ± 0.3, but the pH of 205 mg $CaCO_3$ L^{-1} solution increased by two pH 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 with growth in non-buffered treatments. Subsequently, a MES concentration of 4 mM was found to maintain the pH of 4 and 102 mg $CaCO_3$ 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 $CaCO_3$ L^{-1} was excluded from this study as the 4 mM MES was unable to maintain solution pH within an acceptable range, and MES concentrations >4 mM 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 section 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 8). These results suggest 4 mM 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 µg L^{-1} 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 4 mM 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 8 Population growth of *H. viridissima* exposed Cu and U in the presence and absence of 4 mM MES biological buffer

Metal	EC_{50} (95% CI)	
	MES absent	MES 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 $CaCO_3$ L^{-1}) and pH (6.0 ± 0.3). Raw data for each test-series of a given metal-organism exposure are provided in Riethmuller (2000) 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 10. Summary data for the concentration-response curve are given in Riethmuller (2000) 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 $CaCO_3$ L^{-1}) are given in table 9.

A 25-fold increase in alkalinity (ie from 4.0 to 102 mg $CaCO_3$ L^{-1}) at a hardness of 165 mg $CaCO_3$ 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 9). The trend observed for the EC_{50} values is consistent with the BEC_{10}, MDEC, NOEC and LOEC values given in table 9, such that all endpoints suggest there is no difference in Cu toxicity with a 25-fold increase in alkalinity.

Table 9 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 $CaCO_3$ L^{-1}) and pH (6.0 ± 0.3) conditions, for 96 h

Alkalinity (mg $CaCO_3$ L^{-1})	BEC_{10}	MDEC	NOEC	LOEC	EC_{50} (95% CI)
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)

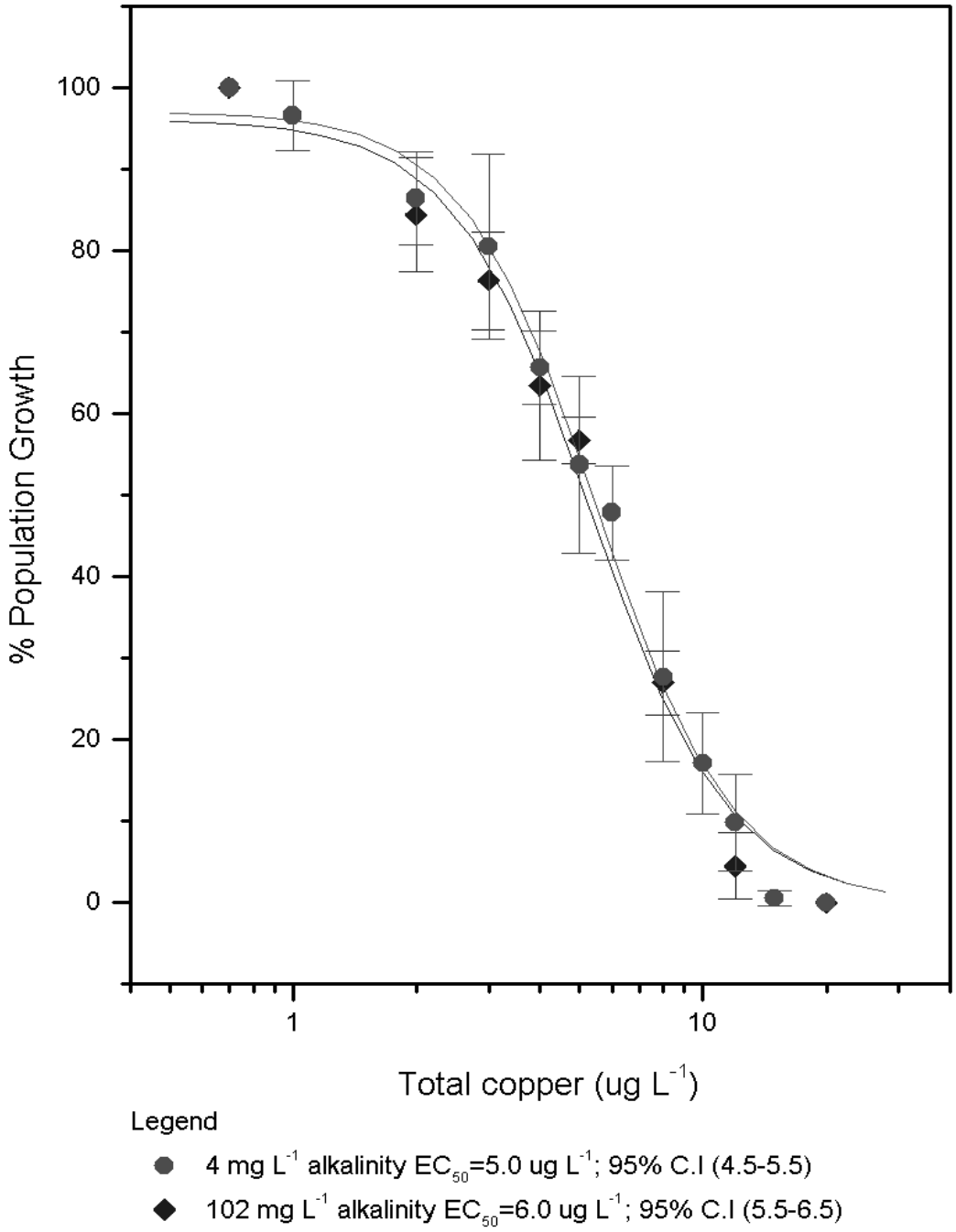

Figure 10 Population growth of *H. viridissima* exposed to Cu over 96 h at two alkalinity levels (4 and 102 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or nine replicates ± 95% CI.

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 $CaCO_3$ L^{-1}) is given in figure 11. 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 $CaCO_3$ L^{-1} alkalinity solution than the 102 mg $CaCO_3$ L^{-1} alkalinity solution (see Riethmuller (2000), Appendix H, table 3).

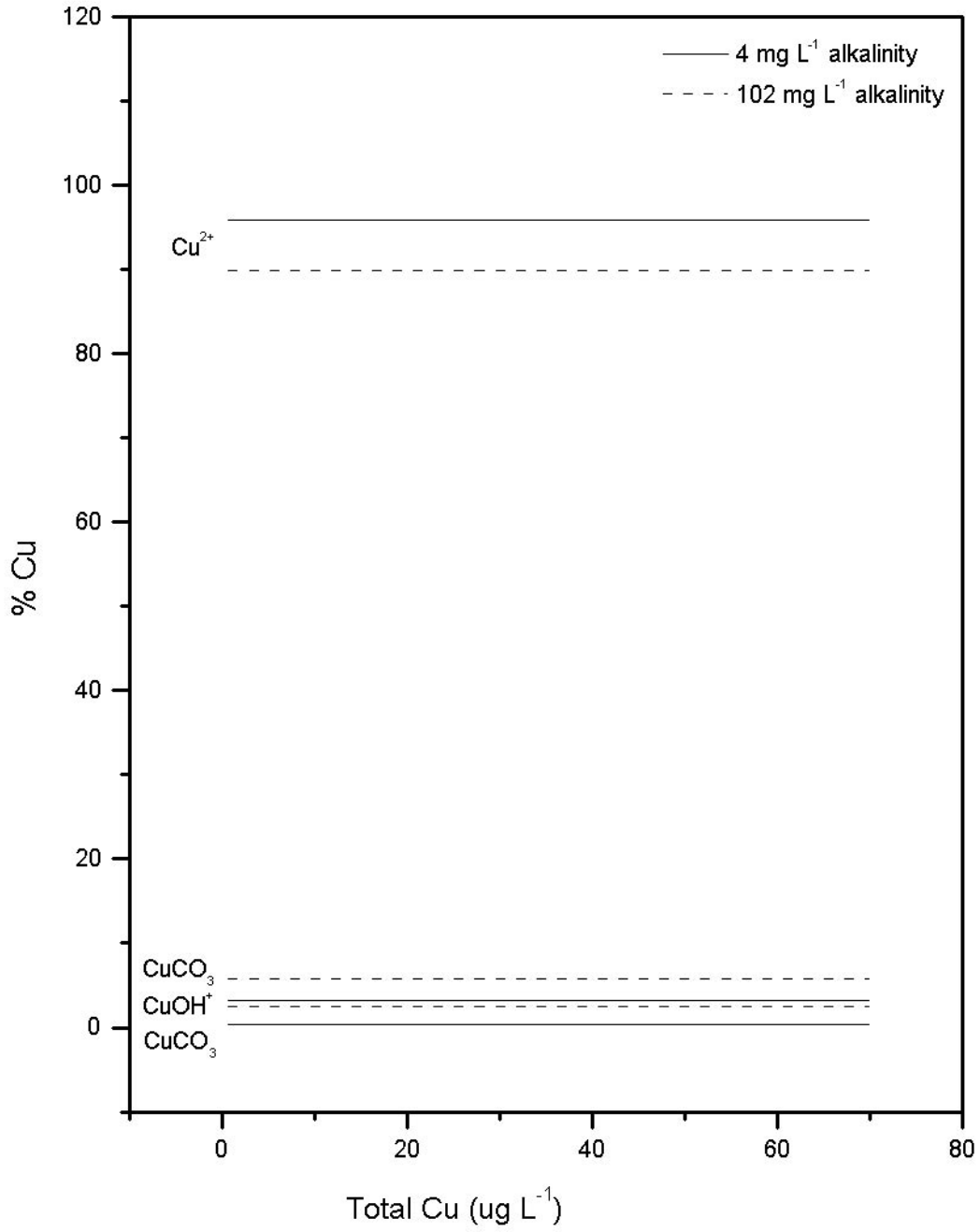

Figure 11 Predicted speciation (% distribution) of Cu in test water (pH 6.0) at two alkalinity levels (4 and 102 mg $CaCO_3$ L^{-1})

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 12. Summary data for the concentration-response curve are given in Riethmuller (2000) 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 $CaCO_3$ L^{-1}) are given in table 10.

Table 10 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 $CaCO_3$ L^{-1}) and pH (6.0 ± 0.3) conditions, for 96 h

Alkalinity (mg $CaCO_3$ L^{-1})	BEC_{10}	MDEC	NOEC	LOEC	EC_{50} (95% CI)
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 $CaCO_3$ L^{-1}), at a hardness of 165 mg $CaCO_3$ L^{-1} 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 10).

The slopes of the two alkalinity concentration-response curves differ, thus precluding a reasonable comparison of the BEC_{10} and MDEC values (fig 12). The BEC_{10} and MDEC at 102 mg $CaCO_3$ L^{-1} alkalinity (25 and 42 µg L^{-1}, respectively) are lower than at 4.0 mg $CaCO_3$ L^{-1} alkalinity (81 and 90 µg L^{-1}, respectively) (table 10), due to the concentration-response curve of the latter having a steeper slope. Note also, that the LOEC at an alkalinity of 102 mg $CaCO_3$ L^{-1} is equivalent to the EC_{50} (table 10).

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 $CaCO_3$ L^{-1}) is given in figure 13. 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, see Riethmuller (2000), Appendix H, table 4), the percentages of UO_2CO_3 increased by a factor of four at 102 mg $CaCO_3$ L^{-1} (compared with 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, $(UO_2)_2(OH)_3CO_3^-$, 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%.

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 to 102 mg $CaCO_3$ L^{-1}, 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.

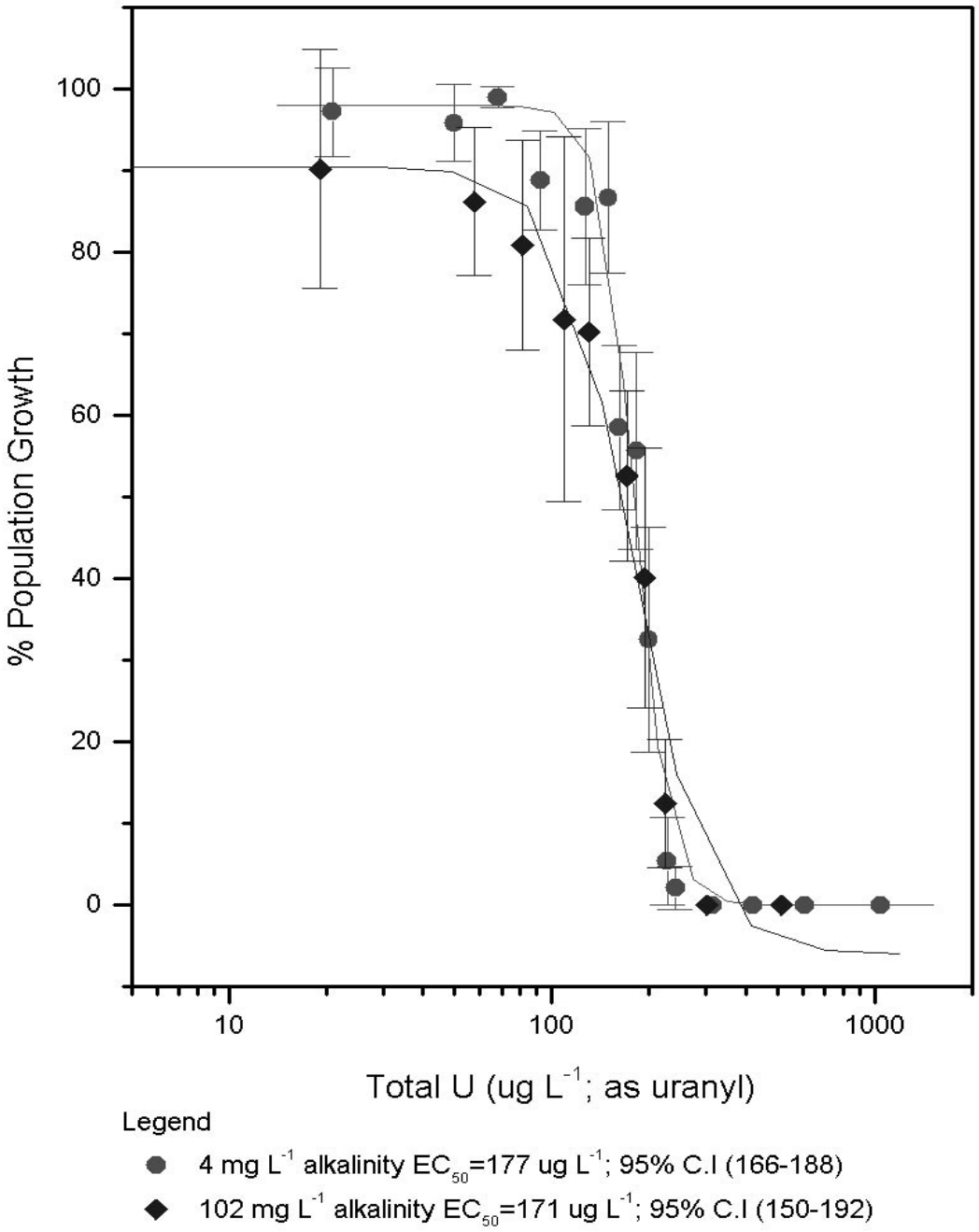

Figure 12 Population growth of *H. viridissima* exposed to U over 96 h at two alkalinity levels (4 and 102 mg $CaCO_3$ L^{-1}). Data points represent the mean of six or nine replicates ± 95% CI.

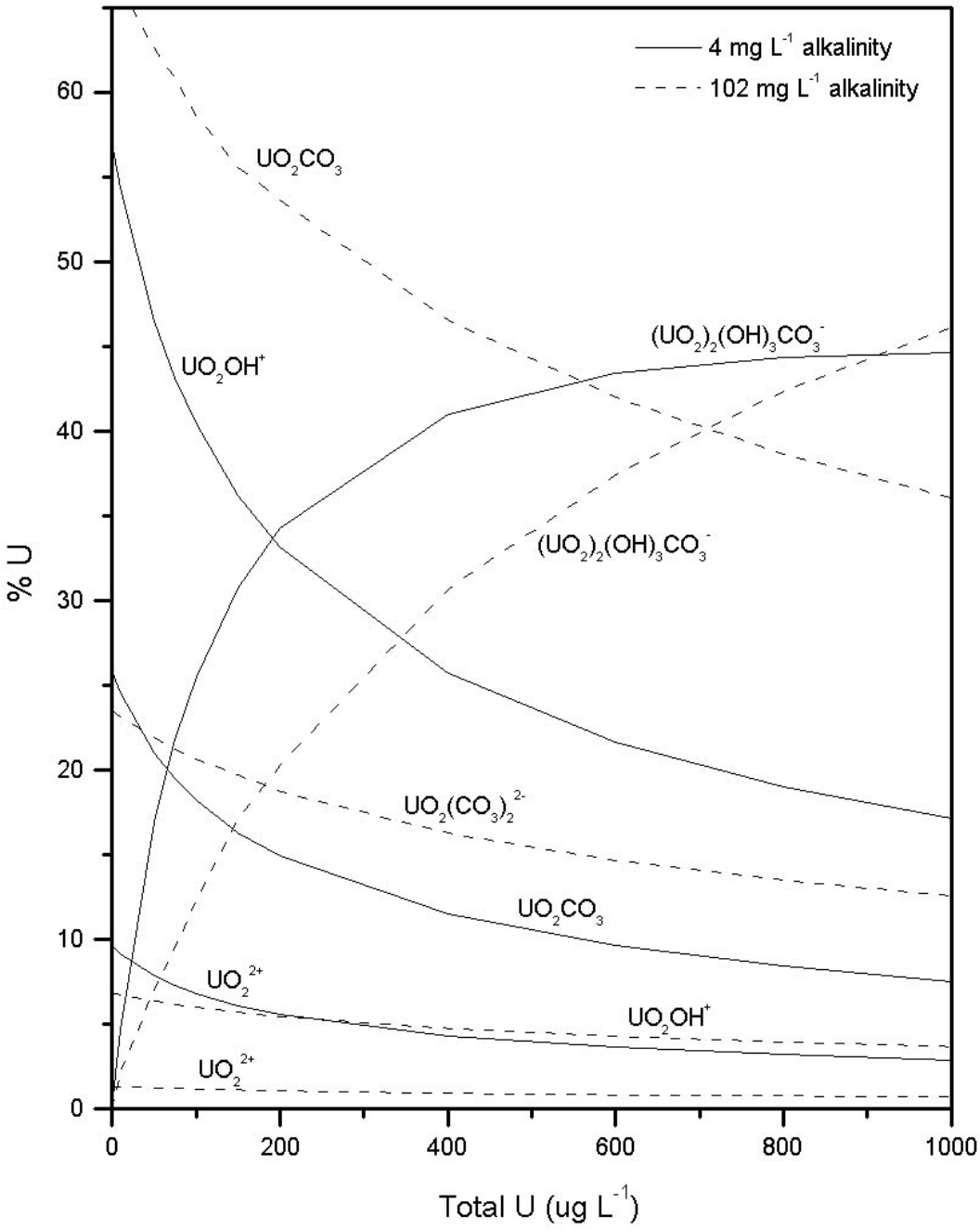

Figure 13 Predicted speciation (% distribution) of U in test water (pH 6.0) at two alkalinity levels (4 and 102 mg $CaCO_3$ L^{-1}). Uranyl species comprising <2% total U are excluded for clarity.

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, supporting 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 (4 mM at pH 6.0; alkalinity 4 and 102 mg $CaCO_3$ 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* population 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, but with 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 2000). 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 & ARMCANZ 2001).

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 & 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 inhibits the uptake and hence toxicity of trace metals at the cell membrane surface (Markich & 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 reduced with increasing water hardness (Miller & Mackay 1980, Mierle 1981, Horne & Dunson 1995 and Erickson et al 1996) and alkalinity (Andrew et al 1977, Miller & Mackay 1980, Laurén & 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 the 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 $CaCO_3$ L^{-1}) and alkalinity (ie 4.0 mg $CaCO_3$ L^{-1}), *H. viridissima* was found to be more sensitive to both Cu and U compared with *M. mogurnda* (tables 11 & 12). 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 11 and 12). 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 1998). 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 with the less sensitive 'lethal' endpoint measuring *M. mogurnda* survival (Hendriks 1995).

Table 11 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[a] (pH 6.0)	Markich & Camilleri (1997)
		13[a] (pH 6.0)	This study

a LC_{50} values cannot be directly compared with the EC_{50} values in this table

Table 12 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}	210 (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 1965[b,c] (pH 6.0)	This study

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

The sensitivity of these organisms to U revealed a similar trend (table 12). 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 210 µg L^{-1} U at pH 5.8 (Markich et al 2000). 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 with the alga, but more than the bivalve. Although the acute response of *M. mogurnda* in this study cannot be directly compared with the chronic response of the other species listed in table 11, 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 12.

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 & Sprague 1985, Part et al 1985, Markich & 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 with 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 & 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 $CaCO_3$ L^{-1}) at a fixed water hardness (165 mg $CaCO_3$ L^{-1}) 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 $CaCO_3$ L^{-1}) 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 & van Ewijk 1993a,b, Denton & Norberg-King 1996, Dhaliwal et al 1997, Moore & 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 summarised 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 & van Ewijk 1993a,b, Chapman et al 1996, Moore & 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 & 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 that can be claimed with 95% confidence that its biological effect does not exceed 10% of the observed effect (Hoekstra & 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 $CaCO_3$ L^{-1} hardness (table 12). 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 $CaCO_3$ 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 $CaCO_3$ L^{-1} hardness is 2-fold less than the NOEC (table 12). 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 low test replication, low number of treatments and large variability in organism response to the herbicide, Tebuthiuron. This trend enabled the same authors to compare the predictive ability of BEC_{10} and NOEC values and conclude 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 & Caux 1997). Ahsanullah and Williams (1991) proposed 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 12). 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 equalled the EC_{50} calculated for U toxicity to *H. viridissima* at 102 mg L^{-1} alkalinity (table 12). It is difficult to confidently use such LOEC values as an estimate of the true lowest adverse biological effect concentration.

The Australian water quality guideline for Cu to protect moderately disturbed freshwater ecosystems is 1.4 µg L^{-1} at a water hardness of 30 mg $CaCO_3$ L^{-1} (ANZECC & ARMCANZ 2001). The Cu guideline value increases as the hardness of the water increases, according to the algorithm given in Section 1.2.1. The hardness modified Cu guideline values at 6.6 and 330 mg $CaCO_3$ L^{-1} are 0.4 and 10.7 µg L^{-1}, respectively. 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 13). These values were not affected by increasing hardness and exceed the guideline value (table 13). *Mogurnda mogurnda* detected Cu at 6.4 and 5.7 µg L^{-1}, at 6.6 and 330 mg $CaCO_3$ L^{-1} hardness, respectively (table 13). The BEC_{10} value at 6.6 mg $CaCO_3$ L^{-1} exceeds the Cu guideline values, but at 330 mg $CaCO_3$ L^{-1} is below the guideline value (table 13). At 165 mg $CaCO_3$ L^{-1} hardness, *M. mogurnda* detected Cu at 9.3 µg L^{-1}, six times greater than the hardness modified Cu guideline (ie 6.0 µg L^{-1}). 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 2000 guidelines.

An interim U guideline for the protection of Australian freshwater ecosystems is 0.5 µg L^{-1} (ANZECC & ARMCANZ 2001). 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 $CaCO_3$ L^{-1} (table 13), which is well above the interim guideline. However, *H. viridissima* detected U at 81 and 47 µg L^{-1} in water with a hardness of 165 and 330 mg $CaCO_3$ L^{-1} respectively (table 13). These BEC_{10} values are approximately 160 times greater than the interim U guideline. *Mogurnda mogurnda* detected U at levels about 2000 times the interim U guideline (ie second investigation, table 13). Unlike the sensitivity of *H. viridissima* to U, increasing hardness did not affect the sensitivity of *M. mogurnda* (table 13). Although the interim 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. Recently, a preliminary site-specific trigger value of 4.9 µg L^{-1} was derived for Magela Creek, based on chronic toxicity data from five local species (R van Dam pers comm). The value, being that predicted to protect 99% of the species (see ANZECC & ARMCANZ 2001), appears more appropriate than the interim trigger value given the historical toxicity data.

Table 13 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 $CaCO_3$ L^{-1})	Alkalinity (mg $CaCO_3$ L^{-1})	BEC_{10}	MDEC	NOEC	LOEC	Effect concentration (95% CI)
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})

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 are 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*, but had no effect 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 & 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 significantly differ with increasing hardness or alkalinity, eliminating it as 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 water 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.

References

Ahsanullah M & Williams AR 1991. Sublethal effects and bioaccumulation of cadmium, chromium, copper and zinc in the marine amphipod, *Allorchestes compressa*. *Marine Biology* 108, 59–65.

Allison HE & Holdway DA 1988. Toxicity of uranium to hydra. In *Alligator Rivers Region Research Institute Annual Research Summary 1987–88,* Australian Government Publishing Service, Canberra, 68–69.

Allison HE, Holdway DA, Hyne RV & Rippon GD 1991. *OSS procedures for the biological testing of waste waters for release into Magela Creek. XII. Hydra test (Hydra viridissima and Hydra vulgaris).* Open File Record 72, Supervising Scientist for the Alligator Rivers Region, Australian Government Publishing Service, Canberra.

APHA (American Public Health Association) 1998. *Standard methods for the examination of water and wastewater.* 20th edn, American Public Health Association, Washington DC, USA.

Andrew RW, Biesinger KE & Glass GE 1977. Effects of inorganic complexing on the toxicity of copper to *Daphnia magna*. *Water Research* 11, 309–315.

Anselme K, Julliard K & Blaineu S 1990. Degradation of metal-labeled collagen implants: Ultrastructural and X-ray microanalysis. *Tissue and Cell* 22, 81–91.

ANZECC 1992. *Australian water quality guidelines for fresh and marine waters.* Australian and New Zealand Environment and Conservation Council, Canberra.

ANZECC & ARMCANZ 2001. *Australian and New Zealand guidelines for fresh and marine water quality*. National Water Quality Management Strategy Paper No 4, Australian and New Zealand Environment and Conservation Council & Agriculture and Resource Management Council of Australia and New Zealand, Canberra.

Apte SC & Day GM 1993. *Organic complexation and partitioning of copper in river waters*: *A review*. OTML/CSIRO Joint report, Sydney.

ASTEC 1993. *Research and technology in tropical Australia and their application to the development of the region.* Australian Government Publishing Service, Canberra.

Azenha M, Vasconcelos MT & Cabral JPS 1995. Organic ligands reduce copper toxicity in *Pseudomonas syringae*. *Environmental Toxicology and Chemistry* 14, 369–373.

Baker L & Walden D 1984. *Acute toxicity of copper and zinc to three fish species from the Alligator Rivers Region.* Technical memorandum 8, Supervising Scientist for the Alligator Rivers Region, AGPS, Canberra.

Bale WM 1919. Further notes on Australian hydroids. IV. *Proceedings of the Royal Society Victoria* 31, 327–361.

Barata C, Baird DJ & Markich SJ 1998. Influence of genetic and environmental factors on the tolerance of *Daphnia magna* (Straus) to essential and non-essential metals. *Aquatic Toxicology* 42, 115–137.

Barnes RD 1980. *Invertebrate zoology.* 4th edn, Saunders College, Philadelphia.

Beach M & Pascoe D 1998. The role of *Hydra vulgaris* in assessing the toxicity of freshwater pollutants. *Water Research* 32, 101–106.

Belanger SE & Cherry DS 1990. Interacting effects of pH acclimation, pH, and heavy metals on acute and chronic toxicity to *Ceriodaphnia dubia* (Cladocera). *Journal of Crustacean Biology* 10, 225–235.

Berlin M & Rudell B 1979. Uranium. In *Handbook on the toxicology of metals,* eds GF Friberg, GF Nordberg & VB Vouk, Elsevier, Amsterdam, 647–658.

Blaise C & Kusui T 1997. Acute toxicity assessment of industrial effluents with a microplate-based *Hydra attenuata* assay. *Environmental Toxicology and Water Quality* 12, 53–60.

Blanquet R & Lenhoff HM 1966. A disulfide-linked collagenous protein of nematocyst capsules. *Science* 154, 152–153.

Boileau LJR, Nieboer E & Richardson DHS 1985. Uranium accumulation in the lichen *Cladonia rangiferina*. Part II. Toxic effects of cationic, neutral, and anionic forms of the uranyl ion. *Canadian Journal of Botany* 63, 390–397.

Borgman U 1983. Metal speciation and toxicity of free metal ions to aquatic biota. In *Aquatic Toxicology,* ed JO Nriagu, John Wiley, Toronto, Canada, 48–72.

Bradley RW & Sprague JB 1985. The influence of pH, water hardness, and alkalinity on the acute lethality of zinc to rainbow trout (*Salmo gairdneri*). *Canadian Journal of Fisheries and Aquatic Sciences* 42, 731–736.

Breault RF, Colman JA, Aiken GR & McKnight D 1996. Copper speciation and binding by organic matter in copper-contaminated streamwater. *Environmental Science and Technology* 30, 3477–3486.

Brown PL, Haworth A, Sharland SM & Tweed CJ 1991. *HARPHRQ: An extended version of the geochemical code PHREEQE.* NSS/R188, UK Atomic Energy Authority, Harwell Laboratory, Oxon.

Brown PL, Markich SJ & Jeffree RA 1994. Migration of uranium: Intergrating geochemistry with biomonitoring to evaluate and predict its environmental impact. *Radiochimica Acta* 66/67, 351–357.

Bryan GW 1976. Some aspects of heavy metal tolerance in aquatic organisms. In *Effects of pollutants on aquatic organisms,* eds APM Lockwood, University of Cambridge Press, Cambridge, 7–34.

Buchwalter DB, Linder G & Curtis LR 1996. Modulation of cupric ion activity by pH and fulvic acid as determinants of toxicity in *Xenopus laevis* embryos and larvae. *Environmental Toxicology and Chemistry* 15, 568–573.

Bywater JF, Banaczkowski R & Bailey M 1991. Sensitivity to uranium of six species of tropical freshwater fishes and four species of cladoceran from northern Australia. *Environmental Toxicology and Chemistry* 10, 1449–1458.

Camilleri C, Markich S, van Dam R & Pfeifle V 1998. *Toxicity of the herbicide Tebuthiuron to Australian tropical freshwater organisms: Towards an ecological risk assessment*. Supervising Scientist Report 131, Supervising Scientist, Canberra.

Campbell PGC 1995. Interactions between trace metals and aquatic organisms: A critique of the Free-ion Activity Model (ch 2). In *Metal speciation and bioavailability in aquatic systems,* eds A Tessier & DR Turner, John Wiley, Chichester, West Sussex, 45–102.

Campbell PGC & Stokes PM 1985. Acidification and toxicity of metals to aquatic biota. *Canadian Journal of Fisheries and Aquatic Sciences* 42, 2034–2049.

Carroll JJ, Ellis SJ & Oliver WO 1979. Influences of hardness constituents on the acute toxicity of cadmium to brook trout (*Salvelinus fontinalis*). *Bulletin of Environmental Contamination and Toxicology* 22, 575–581.

CCREM 1991. *Canadian Water Quality Guidelines*. Environment Canada, Ottawa.

Chapman JC 1995. The role of ecotoxicity testing in assessing water quality. *Australian Journal of Ecology* 20, 20–27.

Chapman PM, Cladwell RS & Chapman PF 1996. A warning: NOECs are inappropriate for regulatory use. *Environmental Toxicology and Chemistry* 15, 77–79.

Choppin GR 1992. The role of natural organics in radionuclide migration in natural aquifer systems. *Radiochimica Acta* 58/59, 113–120.

Crist RH, Oberholser K, Schwartz D, Marzoff J & Ryder D 1988. Interactions of metals and protons with algae. *Environmental Science and Technology* 22, 755–760.

Cusimano RF, Brakke DF & Chapman GA 1986. Effects of pH on the toxicities of cadmium, copper, and zinc to steelhead trout. *Canadian Journal Fisheries and Aquatic Sciences* 43, 1497–1503.

Daly HR, Campbell IC & Hart BT 1990a. Copper toxicity to *Paratya australiensis*: I. Influence of nitrilotriacetic acid and glycine. *Environmental Toxicology and Chemistry* 9, 997–1006.

Daly HR, Campbell IC & Hart BT 1990b. Copper toxicity to *Paratya australiensis*: II. Influence of bicarbonate and ionic strength. *Environmental Toxicology and Chemistry* 9, 1007–1011.

Davies PE, Cook LSJ & Goenarso D 1994. Sublethal responses to pesticides of several species of Australian freshwater fish and crustaceans and rainbow trout. *Environmental Toxicology and Chemistry* 13, 1341–1354.

Denton DL & Norberg-King TJ 1996. Whole effluent toxicity statistics: A regulatory perspective. In *Whole effluent toxicity testing: An evaluation of methods and prediction of receiving system impacts,* eds DR Grothe, KL Dickson & DK Reed-Judkins, SETAC Press, Pensacola, 83–102.

Dhaliwal BS, Dolan RJ, Batts CW & Kelly JM 1997. Warning: Replacing NOECs with point estimates may not solve regulatory contradictions. *Environmental Toxicology and Chemistry* 16, 124–126.

Dzombak DA & Morel FMM 1990. *Surface complexation modeling: Hydrous ferric oxide.* John Wiley, New York.

Ellender G, Jobson S, Hyne RV & Rippon GD 1992. Bioaccumulation of uranium by *Hydra viridissima* and its implications for bioassay. Open file record 94, Supervising Scientist for the Alligator Rivers Region, Canberra. Unpublished paper.

Erickson RJ, Benoi DA, Mattson VR, Nelson Jr HP & Leonard EN 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. *Environmental Toxicology and Chemistry* 15, 181–193.

Flemming CA & Trevors JT 1989. Copper toxicity and chemistry in the environment. *Water, Air and Soil Pollution* 44, 143–505.

Flik G & Verbost PM 1994. Calcium transport in fish gills and intestine. *Journal of Experimental Biology* 184, 17–29.

Florence TM & Batley GE 1980. Chemical speciation in natural waters. *Critical Reviews in Analytical Chemistry* 9, 219–296.

Franklin N, Stauber J, Markich S & Lim R 1998. *A new tropical algal test to assess the toxicity of metals in freshwaters*. Supervising Scientist Report 133, Supervising Scientist, Canberra.

French P 1986. *The speciation and solubility of copper and cadmium in natural waters.* Environmental Report 1226-M, Water Research Centre – Environment, Medmedham, UK.

Gascoyne M 1992. Geochemistry of the Actinides and their daughters. 2nd edn, In *Uranium-series disequilibrium: Applications to earth, marine, and environmental sciences,* eds M Ivanovich & RS Harmon, Clarendon Press, Oxford, 34–61.

Gauss JD, Woods PE, Winner RW & Skillings JH 1985. Acute toxicity of copper to three life stages of *Chironomus tentans* as affected by water hardness-alkalinity. *Environmental Pollution (Series A)* 37, 149–157.

Gerhardt A 1993. Review of impact of heavy metals on stream invertebrates with special emphasis on acid conditions. *Water, Air and Soil Pollution* 66, 289–314.

Giles MS 1974. Toxicity studies on aquatic organisms and grass-sedge communities in the Magela Creek area. In *The Alligator Rivers area fact finding study.* Four AAED Reports, eds PJF Newton, AAEC/E305, Australian Atomic Energy Commission, Sydney.

Good NE, Winget GD, Winter W, Connolly TN, Izawa S & Singh RMM 1966. Hydrogen ion buffers for biological research. *Biochemistry* 5, 467–477.

Greene B, Henzl MT, Hosea JM & Darnall DW 1986. Elimination of bicarbonate interference in the binding of U(VI) in mill-waters to freeze-dried *Chlorella vulgaris*. *Biotechnology and Bioengineering* 28, 746–767.

Grenthe I, Fuger J, Konings RJM, Lemire RJ, Muller AB, Nguyen-Trung C & Wanner H 1992. *Chemical thermodynamics of uranium*. North-Holland, Amsterdam.

Guy RD & Kean AR 1980. Algae as a chemical speciation monitor – I: A comparison of algal growth and computer calculated speciation. *Water Research* 14, 891–899.

Hamelink JL, Landrum PF, Bergman HL & Benson WH (eds) 1994. *Bioavailability: Physical, chemical and biological interactions.* Lewis Publishers, Boca Raton.

Hansten C, Heino M & Pynnonen K 1996. Viability of glochidia of *Anodonta anatina* (Unionidae) exposed to selected metals and chelating agents. *Aquatic Toxicology* 34, 1–12.

Harris JH 1995. The use of fish in ecological assessments. *Australian Journal of Ecology* 20, 65–80.

Hart BT 1981. Trace metal complexing capacity of natural waters: A review. *Environmental Technology Letters* 2, 95–110.

Hart BT, Ottaway EM & Noller BN 1987. Magela Creek system, Northern Australia. I. 1982–83 Wet-season water quality. *Australian Journal of Marine and Freshwater Research* 38, 261–288.

Hendriks AJ 1995. Modeling response of species to microcontaminants: Comparative ecotoxicology by (sub)lethal body burdens as a function of species size and partion ratio of chemicals. *Ecotoxicology and Environmental Safety* 32, 103–130.

Hoekstra JA & van Ewijk PH 1993a. The bounded-effect-concentration as an alternative to the NOEC. *The Science of the Total Environment,* Supplement, 705–711.

Hoekstra JA & van Ewijk PH 1993b. Alternatives for the no-observed-effect level. *Environmental Toxicology and Chemistry* 12, 187–194.

Hogstrand C, Wilson RD, Polger D & Wood CM 1994. Effects of zinc on the kinetics of branchial calcium uptake in freshwater rainbow trout during adaption to waterborne zinc. *Journal of Experimental Biology* 186, 55–74.

Holdway DA 1992. Uranium toxicity to two species of Australian tropical fish. *The Science of the Total Environment* 125, 137–158.

Holdway DA & Wiecek MM 1989. OSS test procedures for the biological testing of waste waters for release into Magela Creek. II: Larval Gudgeon test (*Mogurnda mogurnda*). Open file record 52, Supervising Scientist for the Alligator Rivers Region, Canberra. Unpublished paper.

Horne MT & Dunson WA 1995. Effects of low pH, metals, and water hardness on larval amphibians. *Archives of Environmental Contamination and Toxicology* 29, 500–505.

Howarth RW & Sprague JB 1978. Copper lethality to rainbow trout in waters of various hardness and pH. *Water Resources* 12, 455–462.

Hunt DTE 1987. *Trace metal speciation and toxicity to aquatic organisms – A review.* TR 247, Water Resource Centre – Environment, Marlow, UK.

Hyne RV, Padovan A, Parry DL & Renaud SM 1993. Increased fecundity of the Cladoceran *Moinodaphnia macleayi* on a diet supplemented with a green alga, and its use in uranium toxicity tests. *Australian Journal of Marine and Freshwater Research* 44, 389–399.

Hyne RV, Rippon GD, Hunt SM & Brown GH 1996. *Procedures for the biological toxicity testing of mine waste waters using freshwater organisms*. Supervising Scientist Report 110, Supervising Scientist, Canberra.

Issa AA, Abdel-Basset R & Adam MS 1995. Abolition of heavy metal toxicity on *Kirchneriella lunaris* (Chlorophyta) by calcium. *Annals of Botany* 75, 189–192.

Jackson BP, Lasier PJ, Miller WP & Winger PW 2000. Effects of Ca, Mg and Na on alleviating Cd toxicity to *Hyalella azteca. Bulletin of Environmental Contamination and Toxicology* 64, 279–286.

Jeffree RA & Simpson RD 1986. An experimental study of the uptake and loss of Ra-226 by the tissue of the tropical freshwater mussel *Velesunio angasi* (Sowerby) under varying Ca and Mg water concentrations. *Hydrobiologia* 139, 59–80.

Kennedy PL, Clements WH, Myers OB, Bestgen HT & Jenkin DG 1995. *Evaluation of depleted uranium in the environment at Aberdeen proving grounds, Maryland and Yuma proving grounds, Arizona.* LA-SUB-94-173. Los Alamos National Laboratory, Los Alamos, New Mexico.

Kohler M, Curtis GP, Kent DB & Davis JA 1996. Experimental investigations and modelling of uranium VI transport under variable chemical conditions. *Water Research* 33, 3539–3551.

Kong I-C, Bitton G, Koopman B & Jung K-H 1995. Heavy metal toxicity testing in environmental samples. *Reviews in Environmental Contamination and Toxicology* 142, 119–147.

Lage O, Vasconcelos MTSD, Soares HMVM, Osswald JM, Sansonetty F, Parente AM & Salema R 1996. Suitability of the pH buffers 3-[N–N–bis(hydrxyethyl) amino]–2-hydroxypropanesulfonic acid and N-2-hydroxyethylpiperazine-N'-2-ethane- sulfonic acid for in vitro copper toxicity studies. *Archives of Environmental Contamination and Toxicology* 31, 199–205.

Langmuir D 1978. Uranium solution-mineral equilibria at low temperatures with applications to sedimentary ore deposits. *Geochimica et Cosmochimica Acta* 42, 547–569.

Laurén DJ & McDonald DG 1986. Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri. Canadian Journal of Fisheries and Aquatic Sciences* 43, 1488–1496.

Leckie J & Davis J 1979. Aqueous environmental chemistry of copper. In *Copper in the environment,* ed J Nriagu, Wiley Interscience, New York.

Lesh-Laurie GE 1982. Hydra. In *Developmental biology of freshwater invertebrates,* eds FW Harrison, RR Cowden & R Alan, Liss Inc, New York USA, 69–127.

Macfie SM, Tarmohamed Y & Welbourn PM 1994. Effects of cadmium, cobalt, copper, and nickel on growth of the green alga *Chlamydomonas reinhardtii*: The influence of the cell wall and pH. *Archives of Environmental Contamination and Toxicology* 27, 454–458.

Markich SJ 1998. Effects of biological and physiological variables on the valve movement responses of freshwater bivalves to Mn, U, Cd or Cu. PhD Thesis, University of Technology, Sydney.

Markich SJ & Brown PL 1999. *Thermochemical data for environmentally-relevant elements. 1. Na, K,Ca, Mg, Mn, Fe, Al, U, Cu, Cd, Zn and Pb with model fulvic acid (aspartate, citrate, malonate, salicylate and tricarballyate).* ANSTO/E735. Australian Nuclear Science and Technology Organisation, Sydney.

Markich SJ & Camilleri C 1997. *Investigation of metal toxicity to tropical biota: Recommendations for Revision of the Australian Water Quality Guidelines.* Supervising Scientist Report 127, Supervising Scientist, Canberra.

Markich SJ & Jeffree RA 1994. Absorption of divalent trace metals as analogues of calcium by Australian freshwater bivalves: An explanation of how water hardness reduces metal toxicity. *Aquatic Toxicology* 29, 257–290.

Markich SJ, Brown PL & Jeffree RA 1996. The use of geochemical speciation modelling to predict the impact of uranium to freshwater biota. *Radiochimica Acta* 74, 321–326.

Markich SJ, Brown PL, Batley GE, Apte SC & Stauber JL 2000. Incorporating metal speciation and bioavailabilty into water quality guidelines for protecting aquatic ecosystems. *Australasian Journal of Ecotoxicology* (in press).

Markich SJ, Brown PL, Jeffree RA & Lim RP 2000. Valve movement responses *of Velesunio angasi* (Bivalvia: Hyriidae) to manganese and uranium: An exception to the free ion activity model. *Aquatic Toxicology* 51, 155–175.

Mayer Jr FL, Marking LL, Bills TD & Howe GE 1994. Physicochemical factors affecting toxicity in freshwater: Hardness, pH, and temperature. In *Bioavilability: Physical,*

chemical and biological interactions, eds JL Hamelink, PF Landrum, HL Bergman & WH Benson, Lewis Publishers, Boca Raton, 5–22.

McDonald DG & Wood CM 1993. Branchial mechanisms of acclimation to metals in freshwater fish. In *Fish ecophysiology,* eds JC Rankin & FB Jensen, Chapman and Hall, London, 297–321.

McKinley JP, Zachara JM, Smith SC & Turner GD 1995. The influence of uranyl hydrolysis and multiple site-binding reactions on absorption of U(VI) to montmorillonite. *Clays and Minerals* 43, 586–598.

Meador JP 1991. The interaction of pH, dissolved organic carbon, and total copper in the determination of ionic copper and toxicity. *Aquatic Toxicology* 19, 13–32.

Merrick JR & Schmida GE 1984. *Australian freshwater fish: Biology and management*. Griffin Press, South Australia.

Mierle G 1981. Modification of copper toxicity by other cations. *Journal of Plant Nutrition* 3, 293–308.

Miller TG & Mackay WC 1980. The effects of hardness, alkalinity and pH of test water on the toxicity of Copper to rainbow trout (*Salmo gairdneri*). *Water Resources* 14, 129–133.

Miwa T, Murakami M & Mizuike A 1989. Speciation of copper in fresh waters. *Analytica Chimica Acta* 219, 1–8.

Moore DRJ & Caux P-Y 1997. Estimating low toxic effects. *Environmental Toxicology and Chemistry* 16, 794 –801.

Moore JW & Ramamoorthy S 1984. Copper. In *Heavy metals in natural waters: Applied monitoring and impact assessments,* Springer-Verlag, Berlin, 77–99.

Morse JW & Choppin GR 1991. The chemistry of transuranic elements in natural waters. *Reviews in Aquatic Sciences* 4, 1–22.

Moulin V, Tits J & Ouzounian G 1992. Actinide speciation in the presence of humic substances in natural water conditions. *Radiochimica Acta* 58/59, 179–190.

Nakajima A, Horikoshi T & Sakaguchi T 1979. Ion effects on the uptake of uranium by *Chlorella regularis. Agricultural Biology and Chemistry* 43, 625–629.

Nor YM 1987. Ecotoxicity of copper to aquatic biota: A review. *Environmental Research* 43, 274–282.

NTDTW 1983. *Alligator Rivers Region regional surface water quality monitoring, November 1978 – April 1981.* Northern Territory Department of Transport and Works, Darwin.

O'Shea TA & Mancy KH 1978. The effect of pH and hardness metal ions on the competitive interaction between trace metal ions and inorganic and organic complexing agents found in natural waters. *Water Research* 12, 703–711.

Osmond JK & Ivanovich M 1992. Uranium-series mobilization and surface hydrology. 2nd edn, In *Uranium-series disequilibrium: Applications to earth, marine, and environmental sciences,* eds M Ivanovich & RS Harmon, Clarendon Press, Oxford, 259–289.

Pagenkopf GK 1983. Gill surface interaction model for trace metal toxicity to fishes: Role of complexation, pH and water hardness. *Environmental Science and Technology* 17, 342–347.

Parent L & Campbell PGC 1994. Aluminum bioavailability to the green alga *Chlorella pyrenoidosa* in acidified synthetic soft water. *Environmental Toxicology and Chemistry* 13, 587–598.

Parkhurst BR, Elder RG, Meyer JS, Sanchez DA, Pennak RW & Waller WT 1984. An environmental hazard evaluation of uranium in a Rocky Mountain stream. *Environmental Toxicology and Chemistry* 3, 113–124.

Part P, Svanberg O & Kiessling A 1985. The availability of cadmium to perfused rainbow trout gills in different water qualities. *Water Research* 19, 427–434.

Playle RC, Gresemer RW & Dixon DG 1992. Copper accumulation on gills of flathead minnows: Influence of water hardness, complexation and pH of the gill micro-environment. *Environmental Toxicology and Chemistry* 11, 381–391.

Pollino CA & Holdway DA 1999. The potential of hydra species as standard toxicity test animals. *Ecotoxicology and Environmental Safety* 43, 309–316.

Poston TM, Hanf RW & Simmons MA 1984. Toxicity of uranium to *Daphnia magna. Water, Air and Soil Pollution* 22, 289–298.

Reid SD & McDonald DG 1988. Effects of cadmium, copper, and low pH on ion fluxes in the rainbow trout, *Salmo gairdneri*. *Canadian Journal of Fisheries and Aquatic Sciences* 45, 244–253.

Reid SD & McDonald DG 1991. Metal binding activity of the gills of rainbow trout (*Oncorhychus mykiss*). *Canadian Journal of Fisheries and Aquatic Science* 48, 1061–1068.

Riethmuller N 2000. The effect of true water hardness and alkalinity on the toxicity of Cu and U to two tropical Australian freshwater organisms. Internal Report 348, Supervising Scientist, Canberra.

Rippon GD & Hyne RV 1992. Purple spotted gudgeon: Its use as a standard toxicity test animal in tropical northern Australia. *Bulletin of Environmental Contamination and Toxicology* 49, 471–476.

Rump HH & Krist H 1992. *Laboratory manual for the examination of water, waste water and soil.* 2nd edn, VCH Publications, Weinheim.

Schenck RC, Tessier A & Campbell PGC 1988. The effect of pH on iron and manganese uptake by green alga. *Limnology and Oceanography* 33, 538–550.

Schubauer-Berigan MK, Dierkes JR, Monson PD & Ankley GT 1993. pH-dependent toxicity of Cd, Cu, Ni, Pb and Zn to *Ceriodaphnia dubia*, *Pimephales promelas, Hyalella azteca* and *Lumbriculus variegatus*. *Environmental Toxicology and Chemistry* 12, 1261–1266.

Seefeldt SS, Jenson JE & Fuerst EP 1995. Log-logistic analysis of herbicide dose-response relationships. *Weed Technology* 9, 218–227.

Skidmore JF 1986. Toxicity of copper to selected Australian freshwater animals. In *Water quality management: Freshwater ecotoxicology in Australia,* ed BT Hart, Chisholm Institute of Technology, Melbourne, 41–52.

Skidmore JF & Firth IC 1983. *Acute sensitivity of selected Australian freshwater animals to copper and zinc*. Australian Water Resources Council, Technical Paper No 81, Australian Government Publishing Service, Canberra.

Sorensen EMB 1991. *Metal poisoning in fish.* CRC Press, Boca Raton, Florida.

Stauber JL, Vsai J, Vaughan GT, Peterson SM & Brockbank CI 1994. *Algae as indicators of toxicity of the effluent from bleached eucalypt kraft pulp mills.* National Pulp Mills Research Program. Technical Report No. 3, CSIRO, Canberra.

Stebbing ARD & Pomroy AJ 1978. A sublethal technique for assessing the effects of contaminants using *Hydra littoralis*. *Water Research* 12, 631–635.

Stumm W & Morgan JJ 1981. *Aquatic Chemistry: An introduction emphasizing chemical equilibria in natural waters.* J Wiley, New York.

Sylva RN 1976. The environmental chemistry of copper (II) in aquatic systems. *Water Research* 10, 789–792.

Tarzwell CM & Henderson C 1960. Toxicity of less common metals to fishes. *Industrial Wastes* 5, 12.

Tubbing DMJ, Admiraal W, Cleven RFMJ, Iqbal M, van de Meent D & Verweij W 1994. The contribution of complexed copper to the metabolic inhibition of algae and bacteria in synthetic media and river water. *Water Research* 28, 37–44.

US EPA 1985. *Ambient water quality criteria for copper – 1984.* EPA-440/5-84-031. United States Environmental Protection Agency, Washington DC.

Verbost PM, Lafeber FPJG, Spanings FAT, Aarden EM & Wendelaar Bonga SE 1992. Inhabition of Ca^{2+} uptake in freshwater carp, *Cyprinus carpio*, during short-term exposure to aluminium. *Journal of Experimental Zoology* 262, 247–254.

Waiwood KG & Beamish FWH 1978. The effect of copper, hardness, and pH on the growth of rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology* 13, 591–598.

Welsh PG, Skidmore JF, Spry DJ, Dixon DG, Hodson PV, Hutchinson NJ & Hickie BE 1993. Effect of pH and dissolved organic carbon on the toxicity of copper to larval fathead minnow (*Pimephales promelas*) in natural lake waters of low alkalinity. *Canadian Journal of Fisheries and Aquatic Sciences* 50, 1356–1362.

Williams NJ, Kool KM & Simpson RD 1991. Copper toxicity to fishes and an extremely sensitive shrimp in relation to a potential Australian tropical mining-waste seep. *International Journal of Environmental Studies* 38, 165–180.

Winner RW 1985. Bioaccumulation and toxicity of copper as affected by interactions between humic acid and water hardness. *Water Research* 19, 449–455.

Yong P & Macaskie LE 1995. Role of citrate as a complexing ligand which permits enzymatically-mediated uranyl ion bioaccumulation. *Bulletin of Environmental Contamination and Toxicology* 54, 892–899.