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A new tropical algal test to assess the toxicity of metals in freshwaters

Natasha Franklin,
Jenny Stauber, Scott Markich
& Richard Lim

Environment Australia
Department of the Environment

supervising scientist

Natasha Franklin – Department of Environmental Sciences, University of Technology, Sydney, PO Box 123, Broadway, NSW 2007, Australia. Present address: Centre for Advanced Analytical Chemistry, CSIRO Division of Energy Technology, Private Mail Bag 7, Menai, NSW 2234, Australia

Jenny Stauber – Centre for Advanced Analytical Chemistry, CSIRO Division of Energy Technology, Private Mail Bag 7, Menai, NSW 2234, Australia

Scott Markich – Environment Division, Australian Nuclear Science and Technology Organisation, Private Mail Bag 1, Menai NSW 2234 Australia

Richard Lim – Department of Environmental Sciences, University of Technology, Sydney, PO Box 123, Broadway, NSW 2007, Australia

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Executive summary

Copper (Cu) and uranium (U) are of potential ecotoxicological concern to tropical Australian freshwater biota as a result of mining impacts. No local data on the toxicity of these metals to tropical freshwater algae are currently available. The aim of this study was to develop a toxicity test for an Australian tropical freshwater alga that can be added to the suite of tests currently available for tropical freshwater invertebrates and fish. This toxicity test was used to investigate the toxicity of Cu and U to the alga *Chlorella* sp (new species) in a synthetic softwater and to specifically determine the effect of pH on metal toxicity over the range typically found in soft fresh surface waters in tropical northern Australia.

A growth inhibition toxicity test was successfully developed for this alga, which was isolated from Kakadu National Park, Northern Territory, prior to conducting the toxicity testing. Key environmental parameters including light, temperature and nutrients were optimised to obtain acceptable algal growth rates over 72 hours. HEPES buffer (2 mM at pH 6.5) was found to be a suitable and practical option for pH control that could be incorporated in the test protocol for *Chlorella* sp. The results obtained in this study confirmed a lack of toxic effects by HEPES on the algae, as well as negligible complexation with both Cu and U. Adequate pH control (ie <0.5 pH unit variation over 3 days) was also obtained in the 2 mM HEPES-buffered synthetic softwater.

Based on the minimum detectable effect concentration (MDEC), *Chlorella* sp was more sensitive to Cu (0.7 µg L^{-1}) than U (13 µg L^{-1}), and more sensitive than other Australian tropical freshwater species, with an order of sensitivity: Alga ≥ Crustacea > Cnidaria > Mollusca > Chordata. The toxicity of Cu and U was highly pH-dependent. Copper concentrations needed to inhibit growth by 50% (72 h EC_{50}) increased from 1.5 to 35 µg Cu L^{-1} as the pH decreased from 6.5 to 5.7. The 72 h EC_{50} for U increased from 44 to 78 µg U L^{-1} over the same pH range. Decreased toxicity at pH 5.7 was due to lower concentrations of cell-bound and intracellular Cu and U compared to that at pH 6.5. These results are explained in terms of the possible mechanism of competition between H+ and the metal ion at the cell surface.

The comparative sensitivity of *Chlorella* sp to Cu and U was also assessed. *Chlorella* sp was two times more sensitive to Cu than to U at pH 5.7 and up to 30 times more sensitive to Cu at pH 6.5 on a weight basis. However, on a molar basis, *Chlorella* sp was two times more sensitive to U than to Cu at pH 5.7. At pH 6.5, Cu was >8 times more toxic to the alga than U.

This species was sensitive enough to detect adverse effects of Cu at the ANZECC guideline values of 5 µg Cu L^{-1}, making it a sensitive test organism for the assessment of Cu contamination of freshwaters. However, the unusual, often non-sigmoidal, concentration-response curve for *Chlorella* sp may reduce the reproducibility of the toxicity test. Despite this, *Chlorella* sp does possess a number of desirable characteristics for use in toxicity assessment and therefore is recommended to be used as part of a battery of toxicity tests with other local freshwater organisms. In particular, the alga's high sensitivity to Cu and U and environmental relevance make it a suitable choice for site-specific testing of mine wastewaters in tropical Australia. The findings obtained in this study have the potential to be incorporated into future revisions of the Australian water quality guidelines.

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1 Introduction

1.1 General introduction

Metal contamination of aquatic environments occurs as a result of human activities and affects organisms at the biochemical, cellular, population and community level. Toxicity testing of metals in Australia has been relatively limited, particularly in the tropics, and confined mainly to determining effects on animal species. As such, it has been necessary to use overseas toxicity data to protect Australian biota.

In tropical Australian freshwaters, copper (Cu) and uranium (U) have been identified as priority metals of potential ecotoxicological concern. Their presence is largely the result of mining activities. Although local data on the toxicity of these metals to animals are gradually increasing, there is no information on the toxicity of Cu and U to Australian tropical freshwater algae.

1.2 Toxicity testing using unicellular algae

Toxicity tests with unicellular algae have served a relatively minor role in regulatory decisions concerning the environmental impact of potential pollutants (Lewis 1995). Originally developed to assess eutrophication potential in natural waters (USEPA 1971), algal toxicity tests have been more widely used over the last two decades for toxicity evaluation of industrial wastewaters (Walsh & Merill 1984, Vasseur et al 1991), specific chemicals (Soniassy et al 1977, Klein et al 1993) and sediment leachates (Munawar & Munawar 1987). Despite their increasing use, algal toxicity tests are often considered to be of secondary importance compared to other test organisms (Lewis 1995). Although this opinion is slowly changing, the limited number of toxicity assessments being made with these aquatic plants is not consistent with their ecological significance.

Unicellular algae are the foundation of most aquatic food chains and account for much of the production base of lentic and marine ecosystems. As primary producers, algae fix a major portion of the Earth's carbon and generate, via photosynthesis, much of the oxygen in our atmosphere. Algae constitute a high quality food source in both the oceans and freshwater systems as they are eaten by zooplankton, by vertebrates in their larval stages and by some large marine organisms (Stauber et al 1996). Any adverse impact on algae is likely to affect organisms at higher trophic levels and have important consequences for the health of the whole aquatic ecosystem.

In addition to their aquatic significance, single-celled algae have enormous value as test organisms. Unlike standard, acute toxicity tests with fish or invertebrates, which measure the death of an organism over 96 h, toxicity tests with algae typically measure a sublethal effect, such as inhibition of cell division rate or photosynthesis. This has the advantage of detecting more subtle disturbances as it measures an organism's response to physiological perturbations induced by toxicants, rather than the less subtle effects, such as cell death or disease. Sublethal endpoints with similar sensitivity are also used in animal testing, yet they require much longer periods of exposure. Because algal cells divide once a day, algal toxicity tests are of a chronic nature, as several generations of algal cells are exposed to the toxicant over a 3 d test. These short-term sublethal chronic tests were found to be 6–10 times more sensitive than acute lethality tests with animals (Stauber et al 1994). In addition, algae can be cultured quickly in the laboratory, providing clones of uniform genetic composition for toxicity testing. Moreover, the relative structural simplicity of unicellular algae and their abundance in

nature make them convenient test organisms. In common with other microbial tests, algal toxicity tests are highly reproducible (Stauber et al 1994). Their sensitivity to a wide range of organic and inorganic pollutants (Florence & Stauber 1991, Stauber et al 1994) makes them appropriate test organisms for toxicity evaluation. In spite of these valuable characteristics, toxicity tests using unicellular algae are still conducted less frequently than acute toxicity tests with animal species (Nabholz et al 1993). However with current restrictions by animal ethics committees on the use of fish in acute lethality tests (Stauber 1995), algal toxicity tests will play an increasingly important role in environmental risk assessment in the future.

1.2.1 Growth inhibition toxicity tests

Growth inhibition toxicity tests are the most widely used of all algal tests, and measure decrease in growth rate (cell division rate) or final cell biomass (Stauber 1995). There are various standard algal growth inhibition tests (OECD 1984, Environment Canada 1992), in which healthy exponentially-growing cells are exposed to different concentrations of a toxicant over a 3–4 d period. The original standard algal bottle test (USEPA 1971) was carried out over 14 d; recently, however, there has been a shift towards more short-term tests of 72 h, and even 48 h duration, in order to prevent loss of toxicants by degradation or evaporation (Stauber et al 1994). Algal growth is usually estimated daily by either automatic or manual cell enumeration techniques to determine any stimulatory or inhibitory effects on cell division rate or final cell biomass (Lewis 1995). Finally, growth in each treatment concentration is compared to a control and toxicity expressed as an EC_{50} value (ie the effective concentration to decrease growth by 50%).

1.2.2 Alternative endpoints and their comparative sensitivities

Since toxicants not only inhibit algal growth, but also affect a variety of physiological processes and cellular parameters, alternative algal test endpoints have been used. Of these, inhibition of photosynthesis, commonly based on the ^{14}C-assimilation test or oxygen evolution (Nyholm & Damgaard 1990, Kusk & Nyholm 1991), and respiration (Cedeno-Maldonado & Swader 1974, Stauber & Florence 1987) have received the most attention. Several quantitative comparative studies of metal toxicity to growth, photosynthesis, and respiration have been made for both marine and freshwater species. There is general agreement that growth, commonly measured as cell division rate, is more sensitive than other physiological endpoints. For example, for the freshwater and marine unicellular algae *Chlorella protothecoides*, *Asterionella gracilis* and *Nitzschia closterium* exposed to Cu, growth was more sensitive than photosynthesis, with respiration being the least sensitive process (Lumsden & Florence 1983, Stauber & Florence 1987). Nalewajko and Olaveson (1995) also found growth to be more sensitive to Cu than photosynthesis or respiration in three strains of *Scenedesmus acutus*. From the above studies, the order of sensitivity of these physiological processes in the presence of Cu was:

Growth >> Photosynthesis > Respiration

However, Wong and Chang (1991) found photosynthesis of *Chlorella protothecoides* to be more sensitive than growth in the presence of 100 μg Cu L^{-1}, while Chiaudani and Vighi (1978) found photosynthesis to be the most sensitive endpoint, with growth being the least sensitive, when comparing the sensitivity of growth, ATP, fluorescence and photosynthesis to Cu. The differential sensitivity of test endpoints could be attributed to differences in experimental duration and growth media (Stauber & Florence 1989, Nalewajko & Olaveson 1995).

Other measurable endpoints used in algal toxicity tests include enzyme activity (Stauber & Florence 1990), cellular deflagellation (Winner & Owen 1991), ATP synthesis (Kwan 1989), and a variety of ultrastructural changes (Visviki & Rachlin 1994). These effect parameters are

less reliable and less sensitive compared to growth rate (Stauber & Florence 1987, Visviki & Rachlin 1994). Nevertheless, they are important in studying phytotoxicity as they enhance our understanding of the mechanisms of toxicity of various pollutants.

1.3 Factors affecting toxicity of metals to algae

The results of algal toxicity tests are greatly influenced by the experimental conditions including test medium, incubation conditions, test duration, test inoculum size and water chemistry.

1.3.1 Test medium

A wide range of growth media have been used to culture algae in growth inhibition tests. The majority of experiments reported to date have been carried out in high nutrient culture media (ie the concentration of nutrients greatly exceed those in natural waters). As shown by Lumsden and Florence (1983) and Stauber and Florence (1989), the use of high nutrient culture media in metal toxicity tests can seriously underestimate the toxic effect, as components of the medium can complex or adsorb metals, thereby reducing metal bioavailability and hence toxicity.

Specific media components that influence metal toxicity include phosphate, silicate, EDTA, manganese (Mn), cobalt (Co) and iron (Fe) (Steemann Nielsen & Kamp-Nielsen 1970, Bartlett et al 1974, Florence 1983, Stauber & Florence 1985). Because surface waters contain much lower concentrations of these binding agents (Stauber et al 1996), the use of a high nutrient test medium severely affects the environmental relevance of laboratory-derived results (Lewis 1995). To overcome this problem, recent protocols for toxicity testing with marine and freshwater algae (Stauber et al 1994) recommend the use of natural filtered seawater or synthetic water, enriched only in nitrate and low concentrations of phosphate. Wong and Beaver (1981) earlier proposed that a rich culture medium was necessary in algal toxicity studies to eliminate the problem of nutrient depletion over the test period. Stauber and Florence (1989) later found the use of dilute media was sufficient to support exponential algal growth for up to 96 h. Moreover, the use of low nutrient media maximised the sensitivity in toxicity testing and was much more relevant to natural waters.

Other culture media components that influence metal toxicity include organic molecules, referred to as algal exudates. In general, the bioavailability, and hence toxicity, of metals are reduced by complexation with these algal exudates (van de Berg et al 1979, Starodub et al 1987).

1.3.2 Incubation conditions

The importance of light and temperature for algal growth is well recognised. However, the physiological effect of these factors on metal toxicity has been rarely studied. Of the available literature, research suggests that metal toxicity to algae generally increases as temperature and light intensity increase (Genter 1996).

In order to achieve maximum growth rate, each algal species has a specific optimal temperature. It is not clear whether sub-optimal temperatures increase the vulnerability of algae to metal stress (Nyholm & Kallqvist 1989). Garnharm et al (1992) suggest that decreased metal stress under cooler temperatures is the consequence of inhibited metabolism-dependent uptake mechanisms.

Algal growth increases with light intensity to a saturation level, but little is known about how metal toxicity to algae is influenced by the intensity and quality of light. Stauber (1995) suggested that light intensity may affect the release of photosynthetic products from algal

cells, which in turn may alter the uptake of a toxicant, and subsequently, its toxicity. In addition, light intensity and quality can alter the speciation and toxicity of some metals (eg lead (Pb)) (Genter 1996).

1.3.3 Test duration

The test duration has been found to significantly influence metal toxicity (Thompson & Coulture 1991), largely as a result of pH changes, but also due to alterations in the concentration and behaviour of the test substance. Over the test period, the pH of the medium increases as the algae utilise carbon dioxide and bicarbonate. Such changes may alter the speciation and behaviour of the toxicant, and therefore, its toxicity (Peterson et al 1984). Longer durations of exposure may also result in nutrient depletion, thus making the cells more susceptible to the toxicant. In addition, decreasing toxicity of the test substance may result, due to adsorption to the rapidly increasing algal biomass or to the test container (Lewis 1995). To overcome these limitations, current test protocols now recommend toxicity tests of short duration: typically 48 to 72 h (OECD 1984).

1.3.4 Test inoculum size

The number of cells added initially to the test flasks (ie the test inoculum) has been shown to influence the toxicity of certain metals, including Cu, Pb, zinc (Zn) (Pascucci & Sneddon 1993). In general, the higher the cell density, the less sensitive the algae are to the toxicant due to increased binding of the test substance with the increasing number of cells (Hornstrom 1990). Steemann Nielsen and Kamp-Nielsen (1970) referred to this phenomenon as the 'biomass effect' in their experiments with Cu, concluding that toxicity decreased as the concentration of cells in suspension increased. Most test protocols recommend an inoculum size of $1–4 \times 10^4$ cells mL^{-1}, as it is considered a compromise between maximising sensitivity of the assay, and having sufficient cells to determine cell density daily over 48 to 96 h (Stauber 1995).

1.3.5 Water chemistry

The toxicity of metals is influenced by a variety of water quality parameters including water hardness, pH and organic carbon concentration. Under laboratory conditions pH and water hardness are probably the most influential variables.

Effect of pH

pH is an important factor affecting the toxicity of metals to algae, although, as the literature indicates, a relationship between the two has been difficult to establish. Some authors have shown an increase in metal toxicity with decreasing pH (Starodub et al 1987, Rai et al 1993; 1994) due to the predominance of the free metal ion. It is this free ion that is generally believed to impart toxicity (Baker et al 1983, Borgmann 1983, Starodub et al 1987). Others studies have indicated that the toxicity of metals (eg cadmium (Cd), Cu, and Zn) decreases markedly with decreasing pH (Steemann Nielsen & Kamp-Nielsen 1970, Peterson et al 1984, Campbell & Stokes 1985), the consequence of reduced metal uptake due to competition with H^+ at the cell membrane.

An explanation for these contradictory results may be due to other related factors that also vary with pH, including metal speciation, complexation and precipitation (Peterson et al 1984, Meador 1991). Without controlling the effect of these variables, it is difficult to assess to what extent the reported metal-pH toxicity effects are caused by pH alone (Peterson et al 1984). As such, the effect of pH on metal toxicity depends on the particular metal, and how its speciation changes with pH.

Effect of water hardness

There is a paucity of information on the way in which water hardness (concentration of calcium (Ca) and magnesium (Mg)) affects the toxicity of metals to algae. In the available literature, amelioration of metal toxicity in hard waters has been reported (Whitton 1970, Hutchinson & Collins 1978, Folsom et al 1986, Jayaraj et al 1992). More specifically, there is general agreement that Ca, rather than Mg, is more significant in reducing metal toxicity (Markich & Jeffree 1994).

Hutchinson and Collins (1978) reported that Ca exerts an ameliorative role in metal toxicity, presumably by competing with metal ions for binding sites. An alternative explanation is offered by Rai et al (1981), who suggested that reduced toxicity may be the result of precipitation or complexation by carbonate, bicarbonate, or hydroxides of Ca and Mg. Of the more extensive studies undertaken using animals, it is consistent that a range of metals (Pb, Mn, Cd, Co, Cu) are more toxic to biota in soft rather than in hard waters (Miller & Mackay 1980, Markich & Jeffree 1994).

1.4 Copper

1.4.1 Chemistry and speciation of Cu in natural waters

In natural waters, Cu, like many trace metals, can exist in a wide variety of physico-chemical forms or species (table 1). It is generally accepted that the free cupric ion (Cu^{2+}) is the most toxic form of Cu to aquatic organisms (Sunda & Guillard 1976, Gavis et al 1981, Borgmann 1983, Florence 1983).

Table 1 Physico-chemical forms of Cu in natural waters[a]

Physico-chemical form	General size and form	Example
Simple ionic species	true solution	$Cu(H_20)_6^{2+}$
Weak complexes	(<0.001 µm)	Cu-fulvic acid
Lipid soluble complexes	(<0.001 µm)	Cu-oxinate
Organo-metallic species	(<0.001 µm)	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 Mitrovic (1993)

There is also evidence that complexing agents such as dissolved organic matter (DOM) significantly reduce Cu toxicity and bioavailability, by decreasing the activity of Cu^{2+} (Steemann Nielsen & Wium-Anderson 1970, Stauber & Florence 1989, Apte & Day 1993). The only exceptions to this rule are some lipid-soluble Cu complexes which can rapidly diffuse through the cell membrane and may actually be more toxic than the free metal ion (Florence 1983, Stauber & Florence 1987, Phinney & Bruland 1994).

The speciation of Cu in natural waters is controlled by a number of variables, including pH, organic complexing agents, and inorganic ligands, such as phosphates and carbonates (Markich et al 1997).

In freshwaters, the inorganic speciation of Cu is dependent on pH. At pH <6.0, Cu^{2+} is typically the most abundant species, whereas at pH >7.0, Cu carbonate and hydroxy species are predominant (Peterson et al 1984, Apte & Day 1993). More specifically, Stumm and Morgan (1981) state that the concentration of Cu^{2+} decreases about one order of magnitude

for every 0.5 pH unit above 6. Although ionic Cu is highly correlated with toxicity (Sunda & Guillard 1976, Gavis et al 1981, Borgmann 1983, Florence 1983), some workers have concluded that the Cu hydroxide species may also contribute to toxicity (Cowan 1986, Meador 1991). However, it is difficult to determine the extent to which Cu hydroxides are toxic since pH, a major variable affecting toxicity, and Cu hydroxide concentration cannot be varied independently (Borgmann 1983). There is general agreement, however, that Cu carbonate species are less toxic than other Cu complexes (Borgmann 1983, Hunt 1987).

When trace metal ions, such as Cu^{2+}, are added to natural water they are able to form strong bonds with ligands containing oxygen, nitrogen and sulfur (Hart 1981, Folsom et al 1986). In most natural waters, the concentration of available complexing agents, such as humic substances, is greatly in excess of total dissolved Cu (Folsom et al 1986). As a consequence, most of the Cu in natural waters (90–100%) is present in the form of DOM complexes, and inorganic Cu species comprise only a relatively small portion of the dissolved Cu pool (Florence 1977, Apte & Day 1993, Stauber et al 1996). Copper-DOM complexes are considered to be of low toxicity to aquatic organisms (Stauber et al 1996). The capability of natural organic ligands to reduce the toxic effect of added Cu is generally referred to as the 'complexing capacity' of the water. In most natural waters, Hart (1981) suggests that only a very small portion of this available complexing capacity is used.

1.4.2 Ecotoxicology of Cu to algae

Copper has been found to be one of the most toxic metals to algae. For example, numerous comparative studies have reported the toxicity of Cu to a variety of algal species to be greater than that of Cd (Jayaraj et al 1992, Guanzon et al 1994), Pb (Kessler 1986, Starodub et al 1987), chromium (Cr) (Wong & Chang 1991), nickel (Ni) (Wong & Chang 1991, Jayaraj et al 1992) and thallium (Tl) (Canterford & Canterford 1980). Among the metals listed, Cu is unique in that it plays a dual role as a highly toxic metal and a necessary trace element (Kessler 1986).

In trace amounts, Cu is an essential micronutrient for both algae and higher plants (Sandman 1985). It is an integral part of plastocyanin, a protein involved in the electron transport chain and which also serves as a co-factor for several enzymes, such as polyphenol oxidase (O'Kelley 1974). At concentrations above those required for optimal growth, Cu has been shown to inhibit growth (Aliotta et al 1983, Baker et al 1983, Kessler 1986), extend the lag phase of cultures (Steeman Nielsen & Wium-Anderson 1970, Bartlett et al 1974) and interfere with several important processes including photosynthesis, respiration, pigment synthesis and cell division (Stauber & Florence 1987, Ahmed & Abdel-Basset 1992, Guanzon et al 1994).

1.4.3 Toxicity of Cu to freshwater algae

Freshwater unicellular green algae show considerable variation in their sensitivity towards Cu (table 2). This is largely a consequence of various authors using differing test methodologies. For appropriate comparisons to be made between test results, identical test conditions are required.

The composition of the test medium has a profound effect on the toxicity of Cu. Most studies, including the majority given in table 2, were carried out in high nutrient culture media, containing high concentrations of phosphate, silicate, EDTA, Mn, Co and Fe. It has been shown that these substances complex and adsorb Cu, and hence, reduce its bioavailability and toxicity to unicellular algae (Steemann Nielsen & Kamp-Nielsen 1970, Bartlett et al 1974, Florence et al 1983, Lumsden & Florence 1983, Stauber & Florence 1985).

Table 2 Toxicity of Cu to freshwater unicellular green algae

Algal Species	Test Endpoint	Medium	Test Duration (h)	EC_{50} [a] (µg Cu L^{-1})	pH	Reference
*Chlorella prototheco*ides[b]	Lag time	Basal	–	32×10^4	3.0	Aliotta et al (1983)
*C. prototheco*ides	Lag time	Basal	–	3.2×10^4	6.5	Aliotta et al (1983)
*C. prototheco*ides	Growth inhibition (cell division rate)	Synthetic softwater	72	16	7.5	Stauber & Florence (1989)
*C. prototheco*ides	Growth inhibition (cell division rate)	EPA-AAP	72	24	7.5	Stauber & Florence (1989)
*C. prototheco*ides	Growth inhibition (cell division rate)	MBL	72	>200	7.5	Stauber & Florence (1989)
*C. prototheco*ides	Growth inhibition (cell division rate)	Synthetic hardwater	72	13	7.9	Stauber (1995)
C. vulgaris	Growth inhibition (cell division rate)	Bristols	33 (d)	180	7.0	Rosko & Rachlin (1977)
C. sp 12	Growth inhibition (cell division rate)	Synthetic softwater	72	7.3	7.5	Stauber (unpublished data)
C. stigmatophora	Total cell volume (biomass)	ASW + full SAAM nutrients	21 (d)	70	–	Christensen et al (1979)
Selenastrum capricornutum	Total cell volume (biomass)	SAAM	21 (d)	85	–	Christensen et al (1979)
S. capricornutum	Photosynthetic activity	Algal Assay	24	430	7.7	Turbak et al (1986)
S. capricornutum	Chlorophyll inhibition	Bolds Basal	72–96	6.7×10^4	–	Bozeman et al (1989)
S. capricornutum	Specific growth rate	containing EDTA	72	47	7.5	Nyholm (1990)
S. capricornutum	Photosynthetic activity	Dutch Standard Water	3	8 (free Cu)	8.4	Tubbing et al (1994)
Scenedesmus quadricauda	Photosynthetic activity	CHU–10	4	100	8.0	Starodub et al (1987)
S. quadricauda	Growth inhibition (cell division rate)	CT	24	2.7×10^4	–	Guanzon et al (1994)
S. quadricauda	Photosynthetic activity	CT	24	5.0×10^4	–	Guanzon et al (1994)

a EC_{50} = Cu concentration giving 50% reduction in corresponding test endpoint b *Chlorella prototheco*ides previously named *Chlorella pyrenoidosa*

This is evident in the work of Stauber and Florence (1989), who reported EC_{50} values (effective concentration that reduces algal growth to 50% of the controls) for *Chlorella protothecoides* of 16, 24 and >200 μg Cu L^{-1} in synthetic softwater, an EPA medium and in a high nutrient MBL medium (no EDTA), respectively. The addition of EDTA and Fe further ameliorated the toxic effect of Cu in earlier work by Monahan (1976), who found that 500 μg L^{-1} was needed to cause toxicity to the same species (*Chlorella protothecoides*). Similar reports of EDTA and Fe counteracting the effect of Cu are detailed by Chiaundani and Vighi (1978), Jayaraj et al (1992) and Wong et al (1995).

The interaction of nutrient status with metal toxicity has also received due attention in the literature. It has been proposed that macronutrients, particularly phosphate, play a major role in the regulation of metal toxicity (Bates et al 1985) and there are several reports of decreased toxicity in response to increases in phosphate concentrations (Twiss & Nalewajko 1992, Nalewajko & Olaveson 1995). Aitchison and Butt (1973) state that when the concentration of phosphate in the medium is higher than is nutritionally required by the cell, excess phosphate is taken into the cell and stored in polyphosphate bodies.

Twiss and Nalewajko (1992) found that polyphosphate bodies were able to detoxify Cu in the unicellular green alga *Scenedesmus acutus* by acting as binding sites for intracellular Cu, thereby immobilising it and reducing its toxic potential. Conversely, limiting phosphate concentrations have been found to increase the sensitivity of Cu to *Chlorella vulgaris* (Hall et al 1989). This highlights the need for careful consideration of the nutrient concentrations used for metal toxicity studies.

Another factor which makes comparing toxicity data difficult, is that different algal species excrete varying amounts of Cu chelating compounds, known as algal exudates (van de Berg et al 1979). Under these circumstances, a given concentration of Cu would ultimately be less toxic to a species producing more exudates. Starodub et al (1987) found the presence of algal exudates was effective in ameliorating the toxic effect of 200 μg Cu L^{-1} to the freshwater green alga *Scenedesmus quadricauda*. Some algae may also produce secondary metabolites and proteins intracellularly, referred to as phytochelatins, that similarly act as chelators of trace metals, such as Cu, thereby reducing toxicity (Robinson 1989, Genter 1996). Silverberg et al (1976) demonstrated that it was these intracellular metal chelators that detoxified Cu in the Cu-tolerant strain *Scenedesmus subspicatus*. Furthermore, Robinson (1989) found that the amount of these metabolites produced in *Scenedesmus actutiformis* and *Chlorella fusca* increased following increased exposure to Cu.

The toxicity of Cu to freshwater unicellular algae is also influenced by the presence of other metals. Wong et al (1982) demonstrated that ten metals (arsenic (As), Cd, Cr, Cu, Fe, Pb, mercury (Hg), Ni, silicon (Si), and Zn) at concentrations that were not toxic to algae individually, jointly inhibited primary production of *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. Wong and Chang (1991) found the presence of Cr and Ni, even at very low concentration (100 μg L^{-1}), greatly increased the toxicity of Cu to *Chlorella protothecoides*. Synergism between Mn and Cu (Christensen et al 1979) and antagonism between Cd and Cu (Bartlett et al 1974) have also been reported for the freshwater unicellular alga *Selenastrum capricornutum*.

1.4.4 Mechanisms of Cu toxicity to algae

Metals, such as Cu, are normally transported into an algal cell by the process of facilitated diffusion, involving interactions with membrane proteins (Borgmann 1983). By forming a lipid-soluble complex with a carrier protein on the surface of the cell membrane, Cu can traverse the hydrophobic region of the membrane to be then released inside the cell where it

can elicit a toxic effect (Luoma 1983). In this process, Cu is retained in the cell, but the ligand that was bound to it remains in solution (Stauber et al 1996). The results of Gross et al (1970) suggest that interference with cell membranes is one of the first steps in the sequence of cupric ion toxicity in *Chlorella*. In particular, changes in membrane permeability have been implicated (Sunda & Huntsman 1983). Once inside the cell, Cu may affect cell organelles such as the chloroplast. Wong et al (1994) reported obvious structural alterations such as breakdown of the thylakoidal membranes in the chloroplast of *Chlorella* cells. Stauber and Florence (1987), however, reported no structural changes to the chloroplast of the marine alga *Nitzschia closterium*, following exposure to 200 µg Cu L^{-1}, indicating that this organelle is not the site of Cu action for this species. They suggested that the most likely mechanism of ionic Cu toxicity to *Nitzschia* is that Cu binds and oxidises thiols in the cell, leading to a lowering of the ratio of reduced to oxidised glutathione (GSH/GSSG) and disruption of cell division. Copper may also exert its toxicity by disrupting the production of ATP and inactivating several enzymes, such as catalase (Stauber & Florence 1987).

Copper species that are soluble in the lipid membrane (lipid-soluble metal complexes (LMC)) can diffuse directly through the cell membrane without having to first dissociate. Passive diffusion therefore provides a faster pathway to Cu toxicity (Mitrovic 1993, Stauber et al 1996). The ligand, also transported into the cell by this process, may exert its own toxic effect (Stauber & Florence 1987).

1.5 Uranium

1.5.1 Chemistry and speciation of U in natural waters

In freshwaters, U can exist in many soluble forms, including the dissolved uranyl ion (UO_2^{2+}) and many uranyl complexes such as $(UO_2)_3(OH)_5^+$, $UO_2(CO_3)_2^-$ and $(UO_2(HPO_4)_2)^{2-}$ (Langmuir 1978, Markich et al 1996). The hexavalent uranyl ion state (UO_2^{2+}) is most likely to occur in oxidised surface waters (Langmuir 1978). The speciation of U in natural waters is, like Cu, controlled by a number of variables including pH, organic complexes and inorganic ligands, such as phosphates and carbonates.

The speciation of U is highly dependent on pH. At pH 5 in synthetic softwater, the free hydrated uranyl ion (UO_2^{2+}) is the dominant uranyl species, but is relatively insignificant at pH ≥6 (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 $(UO_2)_3(OH)_7^-$ increases in importance at pH ≥5, particularly at higher U concentrations (Markich et al 1996). Experimental evidence suggests that it is the UO_2^{2+} and UO_2OH^+ species that are responsible for eliciting a toxic response to the freshwater bivalve *V. angasi* (Markich et al 1996).

The inorganic complexation of uranyl ions by carbonates is considered to be of significance in natural waters due to the formation of exceptionally stable complexes (Greene et al 1986). At pH 5–6 in moderate to hard waters, UO_2CO_3 is the dominant U species, while at pH 6–8, $UO_2(CO_3)_3^{4-}$ predominates. The complexation of uranyl by phosphate ions is usually of minor importance in fresh surface waters, in contrast to carbonates.

Dissolved organic matter, in the form of humic substances, is an important and effective metal complexing agent of U in natural waters (Markich et al 1996). As a consequence of this interaction, the uranyl-humate complex has a high propensity for mobilisation in water thereby, reducing its bioavailability, and hence, toxicity to aquatic organisms (Moulin et al 1992, Brown et al 1994). In soft, low-alkaline, organic-rich freshwaters (pH 5–7), uranyl-humate complexes have been identified as the dominant species of dissolved U (Markich et al 1997).

The fate of U in natural waters is also determined by the degree of sorption to a number of substances including clay and mineral particles, Fe and Al(oxy)hydroxides, silica and microorganisms (Greene et al 1986, McKinley et al 1995, Kohler et al 1996). This interaction reduces the mobility of U, thereby decreasing its potential hazard to aquatic organisms.

1.5.2 Ecotoxicology of U to freshwater algae

No data on the toxicity of U to algae are available. A review of U ecotoxicological data for freshwater animals, however, has recently been given by Markich and Camilleri (1997). Using similar water chemistry, comparable sensitivities were reported for the tropical freshwater bivalve *Velesunio angasi* (48 h EC_{50} value of 144 µg L^{-1} as UO_2) and the green hydra *Hydra viridissima* (96 h EC_{50} value of 108 µg L^{-1} as UO_2). The gudgeon fish *Mogurnda mogurnda* had a 48 h LC_{50} value of 1298 µg L^{-1} as UO_2.

Studies conducted on the toxicity of U to aquatic organisms have reported amelioration of toxicity due to complexation (eg uranyl-humate complexes) or competition with hardness ions (Ca^{2+} and Mg^{2+}) (Parkhurst et al 1984, Poston et al 1984, Barata et al 1998). Markich et al (1996) showed that the toxicity of U was inversely related to pH over the range 5−6, and that UO_2^{2+} and UO_2OH^+ were the most toxic species.

1.6 Significance of water quality guidelines for protecting aquatic ecosystems

The current Australian water quality guidelines (ANZECC 1992), based primarily on laboratory toxicity data, provide a framework to assess the water quality required to protect aquatic ecosystems (Chapman 1995).

Because limited Australian toxicity data are available, the current water quality guidelines are based predominantly on overseas toxicity data using Northern Hemisphere species. The fundamental question of whether this data set is relevant to protecting Australian species is frequently raised and increasingly scrutinised, particularly since many of the species used in the overseas toxicity studies are not native to Australia.

The inability of overseas toxicity data to reflect Australian environmental conditions is perhaps the most obvious shortcoming of the current guidelines. With regard to factors such as temperature, turbidity, dissolved oxygen and pH, Australian freshwaters differ markedly from world averages (Firth 1981). Moreover, variable water flow, low alkalinity and hardness, and high water colour are distinct features of the Australian freshwater environment (Skidmore & Firth 1983). Because all of these environmental factors may modify toxicity, the ability of the current Australian guidelines to adequately protect the aquatic environment cannot be assured. More specifically, the suitability of the current guidelines (ANZECC 1992), derived from a cool temperate climate, to tropical Australia is largely unknown. This issue is particularly relevant since the tropical zone comprises approximately 40% of the Australian continent (ASTEC 1993).

A dearth of information currently exists on the toxicity and environmental effects of potential pollutants to tropical Australian freshwaters. Of potential ecotoxicological concern to biota inhabiting freshwater systems of tropical Australia are mine wastewaters and their constituents (Markich & Camilleri 1997). In particular, U and Cu have been identified as being of environmental significance (Markich & Camilleri 1997). Water hardness, pH and organic carbon all modify the toxicity of these metals by either increasing or decreasing the concentration required to produce a particular biological response (Chapman 1995). However,

these water quality parameters have not been used quantitatively to modify guideline values for metals.

Furthermore, the ability of the current guidelines to adequately protect Australian freshwaters, particularly in the tropics, is even more questionable considering a guideline for U does not currently exist. This is a noticeable shortcoming, given that Australia has a large proportion of the western world's uranium reserves (Minerals Council of Australia 1998), as well as current and future interest in uranium mining, particularly in the tropics.

1.7 Aims and objectives of the study

The development of a strategy for water quality management in tropical Australia is dependent on the quality of available ecotoxicological data on local species. To this end, the aim of the study was to develop a tropical algal toxicity test using the freshwater unicellular alga *Chlorella* sp, isolated from Kakadu National Park, Northern Territory. The algal test was used to produce relevant toxicity data for Cu and U for possible inclusion in the Australian water quality guidelines for the protection of aquatic ecosystems. These guidelines are currently being revised as part of a National strategy to improve the quality of water resources in Australia and New Zealand.

The specific objectives of the study were:

1 To establish suitable toxicity test conditions for the growth of *Chlorella* sp (new species) in a synthetic softwater, by altering key variables such as nutrient composition and light intensity.

2 To compare the effect of Cu and U on the exponential growth rate (cell division rate) of *Chlorella* sp using the new toxicity test.

3 To determine the effect of pH on the toxicity of Cu and U to *Chlorella* sp.

2 Materials and methods

2.1 General analytical procedures

All solutions, including the culture medium, were prepared with high purity Milli-Q water (>18 MΩ cm^{-1} resistivity). To avoid metal contamination, all glassware was soaked in 10% (v/v) nitric acid (HNO3) overnight and was thoroughly rinsed with Milli-Q water prior to use. All polycarbonate vials used for chemical analysis were acid-washed and rinsed in the same manner, except those used for nitrate and phosphate analysis, which were soaked overnight in 10% (v/v) hydrochloric acid (HCl). All reagents used were analytical grade except for ultra-pure HNO3 (Normatom).

2.2 Test organism

2.2.1 Test species

The unicellular freshwater green alga *Chlorella* sp (Chlorophyceae) was isolated from surface water collected within Kakadu National Park, Northern Territory (fig 1) (Padovan 1992) and maintained at the Environmental Research Institute of the Supervising Scientist (***eriss***). Identification to species level was attempted by Dr H Ling from Deakin University, who concluded that this isolate was a new *Chlorella* species. The culture was sent to the laboratories of the CSIRO Division of Energy Technology, Sydney, where it was made axenic (bacteria free) prior to commencement of this research project.

2.2.2 Stock culture maintenance

The alga was cultured axenically in a modified MBL medium (Stein 1973) (table 3). Algae were aseptically transferred into a sterile liquid growth medium in a 250 mL Erlenmeyer flask using a disposable sterile glass pipette on a weekly basis.

The stock cultures were incubated at 27 ± 1°C on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 75 µmol photons PAR $m^{-2}s^{-1}$).

Growth of this algal species was initially monitored over a 21 d period to identify the growth phases under batch culture conditions. A known aliquot of cells ($2–4 \times 10^4$ cells mL^{-1}) was inoculated into 50 mL of MBL medium and counted periodically by both manual and electronic cell enumeration techniques (see section 2.3.4). The shape of the growth curve was then used to determine an appropriate inoculum age for the toxicity test. Exponentially growing cells (<5 d old) were used in all algal tests to avoid the lag period of growth at the beginning of the test. Algal cultures were transferred on a weekly basis to ensure a regular supply of exponentially growing cells.

Routine microscope examination of the algal stock culture, using a phase contrast microscope, was conducted to ensure good cell morphology and the absence of contaminants. Routine sterility checks were also carried out throughout the period of study following the procedure described in section 2.2.3.

Table 3 Final concentration of nutrients in the culture medium for stock culture maintenance of *Chlorella* sp

Nutrient		**MBL medium**
Major constituents		**Concentration (mg L^{-1})**
Tris		500
$NaNO_3$		83
$NaHCO_3$		13
K_2HPO_4		8.7
Ca^{2+}		10
Mg^{2+}		6.6
Cl^-		7.9
SO_4^{2-}		4.8
Na_2EDTA		4.4
Minor Constituents		**Concentration (µg L^{-1})**
Si^{4+}		1.0
Mn^{2+}		50
Zn^{2+}		5.0
Co^{2+}		2.2
Cu^{2+}		2.3
Fe^{3+}		152
Vitamins	– Thiamine	200
	– Biotin	1.0
	– B12	1.0

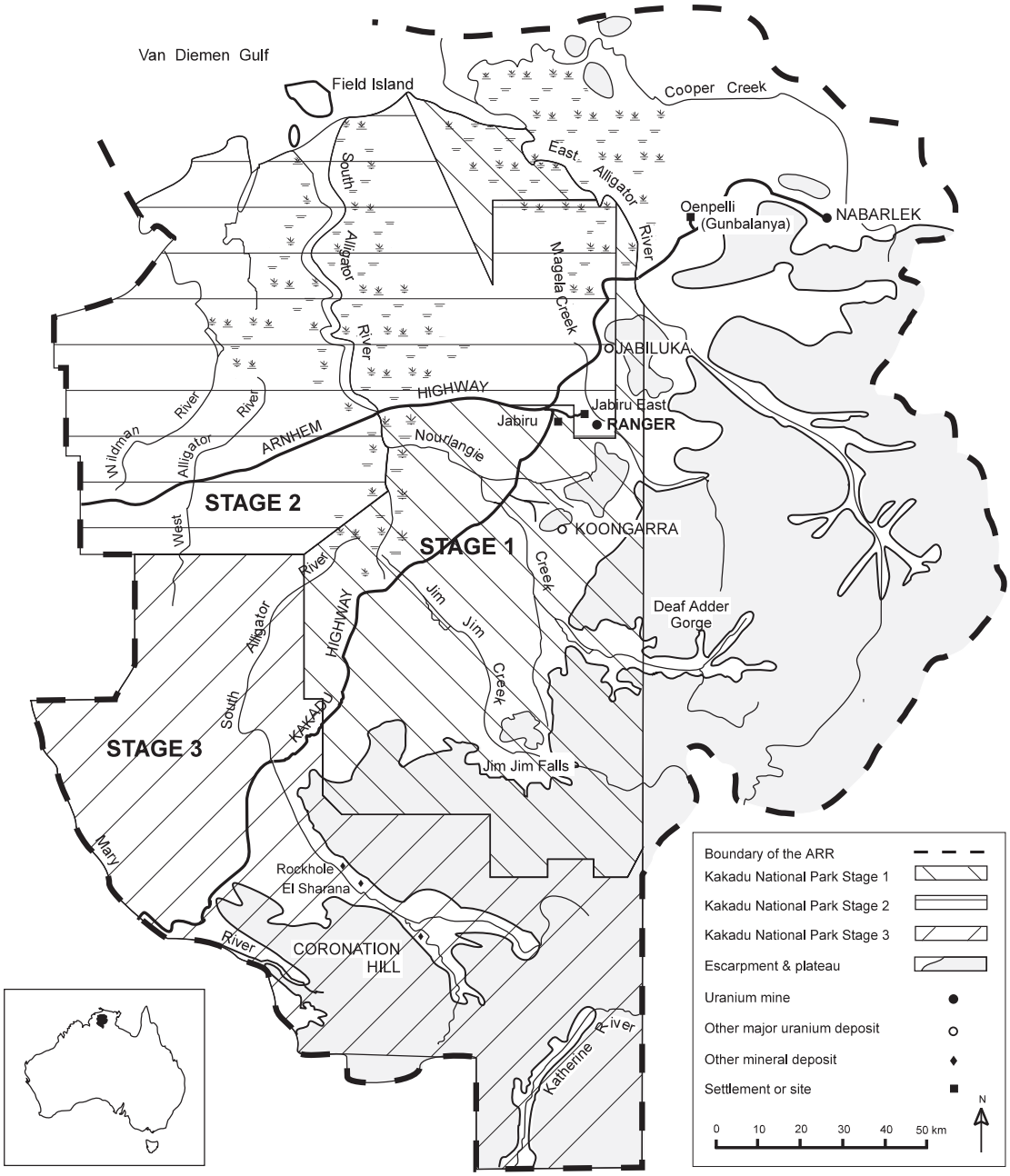


Figure 1 Alligator Rivers Region

2.2.3 Isolation of alga to remove bacteria

The culture was streaked onto MBL agar using a sterile plastic loop in a laminar flow hood and then incubated at 27 ± 1°C on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 75 µmol photons PAR $m^{-2}s^{-1}$) for approximately one week, or until algal colonies were visible. A discrete algal colony was then picked off using a sterile plastic loop and streaked onto a new agar plate to isolate the algae from the bacteria. The agar plate was then incubated for another week and the process repeated until a pure culture was established. A sterility check was performed by examining the culture under a phase contrast microscope and by plating the culture onto peptone yeast extract agar (PYEA) (appendix B). The agar plate was then incubated in the dark at 27°C for one week. This medium promotes the rapid

growth of freshwater bacteria if present in the culture. Absence of bacteria after incubation is indicative of a bacteria free culture.

2.3 Initial toxicity test procedure

The first stage of this research was to develop a toxicity test based on growth inhibition of *Chlorella* sp (new species). Prior to setting up the test protocol, initial test procedures such as preparation of the algal inoculum and test solutions, and algal cell counts were based on an existing test protocol for the temperate freshwater alga *Chlorella protothecoides* (Stauber et al 1994).

2.3.1 Test solution (control/dilution water)

Tests were conducted in a synthetic softwater with a chemical composition similar to that of sandy braided streams in tropical Northern Australia during the wet season. Specifically, water quality characteristics were based on the water of Magela Creek (Alligator Rivers Region, Northern Territory, fig 1). This water is very soft (2–4 mg $CaCO_3$ L^{-1}) and slightly acidic (pH 6.0), with very low concentrations of ions (table 4).

Table 4 Concentration of inorganic components in synthetic and real Magela Creek water

Physico-chemical parameter	Nominal concentration in synthetic water	Measured concentration in real water[a]
pH	6.0 ± 0.5	6.0 ± 0.5
Temperature (°C)	27 ± 1	28
Na (mg L^{-1})	1.00	0.99
K (mg L^{-1})	0.37	0.37
Ca (mg L^{-1})	0.45	0.45
Mg (mg L^{-1})	0.60	0.60
Cl (mg L^{-1})	2.32	1.33
SO_4 (mg L^{-1})	3.12	0.24
HCO_3 (mg L^{-1})	2.63	2.6
NO_3 (µg L^{-1})	15.2	15.2
PO_4 (µg L^{-1})	7.0	7.0
Fe (µg L^{-1})	100	90
Al (µg L^{-1})	70	70
Mn (µg L^{-1})	9.7	9.7
U (µg L^{-1})	0.10	0.110
Cu (µg L^{-1})	0.70	0.70
Zn (µg L^{-1})	0.70	0.30

[a] data from Markich (unpublished)

The use of a synthetic softwater serves as a useful benchmark for providing a maximum risk scenario with respect to assessing the potential impact of metals to aquatic biota. This is because the synthetic water lacked any organic chelating agents (ie dissolved organic carbon DOC) that are present in natural waters. When metals form complexes with DOC, their toxicity to freshwater biota can be substantially ameliorated (Meador 1991).

The synthetic softwater was prepared in 5 L volumes and pH adjusted to pH 6.0 with 0.02 M NaOH. The softwater was then filtered through a Millipore HA 0.45 µm membrane filter. An

aged and acid-washed 5 L plastic container was used to store the water at 4°C for no longer than two weeks before use.

2.3.2 Preparation of the algal inoculum

The algal inoculum was prepared 2–3 h before incubation in the toxicity test. The inoculum was composed of exponentially growing *Chlorella* cells harvested from a 4–5 d old stock culture.

The algal cells were centrifuged in 30 mL glass centrifuge tubes at 2500 revolutions per minute (rpm) at 20°C in a Joun CR4.11 refrigerated centrifuge for 7 min. The nutrient medium (supernatant) was decanted and the cell pellet re-suspended in about 30 mL of Milli-Q water by gentle vortexing. The centrifugation and washing procedure was repeated three times to remove the high nutrient culture medium, which would otherwise ameliorate toxicity due to its ability to strongly complex trace metals (Stauber & Florence 1989). The cell pellet was finally re-suspended in about 15 mL of Milli-Q water.

The re-suspended cells were gently homogenised in a hand-held glass tissue grinder with teflon pestle to disaggregate cell clumps. An aliquot of cell suspension (inoculum 0.1 mL) was added to a 250 mL flask containing 50 mL of synthetic softwater. The cell density in this flask was determined microscopically using a haemocytometer (see section 2.3.4). The desired inoculum was calculated to give a final cell density of $2–4 \times 10^4$ cells mL^{-1} in the test flask.

2.3.3 Preparation of test solutions

The flasks used for all toxicity tests were 250 mL Erlenmeyer flasks coated with a silanizing solution, Coatasil (Ajax), and acid-washed immediately before use. Silanisation of the flasks was essential to minimise the adsorption of metals to the glass thereby reducing their bioavailability, and possibly toxicity, to the algae during the test. For all flasks, 0.5 mL of 26 mM sodium nitrate (15 mg NO_3 L^{-1}) and 0.05 mL of 1.3 mM potassium dihydrogen phosphate (0.15 mg PO_4 L^{-1}) was added to 50 mL of filtered synthetic softwater. Stauber et al (1994) found these to be the only nutrients required to maintain exponential growth over a 3 d test for the temperate freshwater unicellular alga *Chlorella protothecoides*.

Triplicate controls, together with at least five test concentrations were inoculated with $2–4 \times 10^4$ cells mL^{-1} of prewashed *Chlorella* sp cells. In all tests, *Chlorella* sp cells less than approximately 4–5 d old were utilised as the test organisms. Flasks were incubated at 27 ± 1°C on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 120 µmol photons PAR $m^{-2}s^{-1}$). As standard practice, all assay flasks were shaken twice daily by hand to avoid gas limitation. This consisted of swirling the solution approximately six times in the clockwise direction and six times in the anti-clockwise direction. On days 0 and 3 of the toxicity test, the pH was measured in one replicate flask from each test treatment.

2.3.4 Algal cell counts

Cell density in each flask was determined daily for three days. The toxicity test counting technique primarily involved electronic cell enumeration, as it is faster and more precise than manual methods (Stauber et al 1994). Manual counts were performed in conjunction with electronic counts for a number of preliminary experiments to ensure that the electronic counter was accurately determining cell numbers for the test species.

Electronic cell enumeration

A Coulter Multisizer II Particle Analyser with a 70 µm aperture was used in the narrow mode with window settings ranging from 1.45–9.36. Before counting, the test flasks were well mixed by swirling the solution six times in a clockwise direction and six times in an anti-clockwise direction. An aliquot of cells (2.5 mL) was immediately taken from each flask and

diluted to 10 mL with 0.45 μm filtered seawater. This dilution in seawater was necessary to provide sufficient electrolyte for electronic particle counting. The sample was homogenised in a tissue grinder to break up cell clumps prior to counting. Four 100 μL aliquots were counted for each flask and the mean count ± 2 standard deviations (SD) was determined.

Each day, a background count (1:4 dilution with seawater) from a test flask containing no algal cells was determined. This 'Coulter blank', arising from small particles other than algal cells, was then subtracted from the mean algal cell count for each flask.

Coulter calibration

One problem associated with electronic particle counting is that at high cell densities, two cells may be counted as one cell. Although the Coulter Counter has automatic correction for this (coincidence correction), at very high cell densities the instrument tends to underestimate cell densities. It is therefore important to perform a calibration experiment to determine over what range of cell densities the Coulter Counter gives an accurate count. This calibration experiment was performed on a series of dilutions of *Chlorella* in seawater, ranging from 1:5 to 1:600, to compare:

a) Coulter counts with microscope counts

b) Coulter counts with theoretical counts

The theoretical counts were determined by dividing the highest microscope count by the appropriate dilution factor. Linear regressions were performed on both a) and b) to determine the appropriate range of cell densities for routine counting.

Manual cell enumeration

Algal cells were counted using phase contrast optics and a haemocytometer. Although this method is less precise than the electronic counting method, it does permit the direct examination of the cell morphology and is necessary on day 0 when cell densities are low.

Sub-samples of cells from each flask (1−2 mL) were taken and homogenised in a tissue grinder to break up cell clumps. No dilution with seawater or background count correction was required.

2.3.5 Test endpoints and calculations

The growth rate (cell division rate) for each flask over 72 h was calculated using linear regression analysis. A regression line was plotted for $\log_{10}$ cell density vs time (h) to determine the slope of the line for each flask, which is equivalent to the cell division rate per h (μ) for each treatment. Daily doubling times were calculated by multiplying this value $\mu \times 24 \times 3.32$ (constant). A test acceptability measure (mean cell division rate in controls ± 2 SD) was derived from the results of all optimisation tests. Test results were acceptable if the control growth rates were within this range (1.40 ± 0.3 doublings d^{-1}). Growth rates of the treated flasks were presented as a percentage of the control growth rate. A calibration curve was then fitted to the data by plotting the percentage control growth rates vs the measured toxicant concentrations.

The endpoints of the algal growth inhibition test were the 72 h EC_{50} and the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC). The EC_{50} is the effective concentration giving 50% reduction in algal growth rate over 72 h compared to the controls. This was calculated using Trimmed Spearman-Karber analysis (Hamilton et al 1977). Estimates of EC_{25} and EC_{75} values were also calculated using Maximum Likelihood Probit analysis (ToxCalc. Version 5.0.14, Tidepool Software). After testing the data for normality and homogeneity of variances, Dunnett's Multiple Comparison Test was used to

determine which treatments were significantly different from one another. This information enabled estimation of the LOEC and NOEC. Alternative statistical measures to the NOEC and LOEC were also calculated. The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, was estimated using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, was estimated using the approach described by Ahsanullah and Williams (1991).

2.4 Optimisation of the algal toxicity test

A series of 72 h growth inhibition toxicity tests were performed to identify the best growth conditions for *Chlorella* sp tests in a low nutrient softwater. Toxicity tests were conducted using 2 to 3 replicates per treatment, following the standard test procedure described in section 2.3. The effect of nutrient concentration (nitrate and phosphate), light intensity, shaking and pH on the growth of *Chlorella* sp over 72 h was investigated in order to optimise control growth conditions for the toxicity test.

2.4.1 Nutrients

Growth of *Chlorella* sp at various nitrate concentrations (1–20 mg $NO_3\ L^{-1}$) was tested while keeping the phosphate concentration constant at 0.15 mg $PO_4\ L^{-1}$. This level of phosphate was chosen as it is the standard concentration used for 72 h algal toxicity tests with temperate Australian species (Stauber et al 1994). A stock solution of sodium nitrate (26 mM) and potassium dihydrogen phosphate (1.3 mM) was prepared and aliquots added to the test flasks.

The effect of phosphate concentration on algal growth was also determined. Phosphate concentrations ranging from <18 µg $PO_4\ L^{-1}$ (background concentration of synthetic water) to 1.5 mg $PO_4\ L^{-1}$ were tested while maintaining a constant nitrate concentration of 15 mg $NO_3\ L^{-1}$. Similarly, this level of nitrate was chosen as it is the standard concentration used for 72 h algal toxicity tests in Australia (Stauber et al 1994).

2.4.2 Light intensity

Growth (cell division rate) of *Chlorella* sp was investigated under conditions of varying light intensity: 35, 72, 120 µmol photons PAR $m^{-2}s^{-1}$, as determined using a Lic-Cor Model 185 light meter. The concentration of nitrate (15 mg $NO_3\ L^{-1}$) and phosphate (0.15 mg $PO_4\ L^{-1}$) added to the test medium was determined from the results of the nutrient optimisation tests.

2.4.3 Gas exchange: Mini-scale toxicity test

In order to test the effect of continuous shaking on the growth rate of the algal controls over the 72 h toxicity test, it was necessary to miniaturise the test to fit the mechanical shaker. Shaking the flasks promotes gas exchange and ensures that the medium does not become carbon dioxide limited.

The toxicity test was carried out in clean acid-washed 22 mL glass vials which were coated with silanising solution (Coatasil). These vials were used once and discarded. Each vial contained 3 mL of synthetic softwater. The addition of nitrate and phosphate and the algal inoculum was determined as for the test flasks and then scaled down to 3 mL volumes. Each treatment (shaking/non shaking) comprised three vials.

Three vials were shaken on a Bio-Line Orbital shaker at 27°C at a light intensity of 70–75 µmol photons PAR $m^{-2}s^{-1}$. Another three vials were positioned adjacent to the shaker. A flask control was maintained under identical conditions to the non-shaken vials to allow a comparison of cell division rates using the flask and mini-vial test methods. An additional two vials were prepared for each treatment to record the pH on day 0 and day 3.

2.4.4 pH

While initial tests confirmed that this species grew well at pH 6.0 (see section 3.4), a lower pH of the test water was required to cover the range of conditions typical of Australian tropical freshwaters. A toxicity test was set up to investigate the growth of *Chlorella* sp at pH 5.7 and pH 6.0. To avoid changes in pH that occur over the test period as a result of the utilisation of carbon dioxide and bicarbonate and the uptake of nitrogen by algal cells, test flasks were adjusted to their original pH (either pH 5.7 or pH 6.0) daily by the addition of 0.02 M NaOH or 0.01 M HCl. A corresponding set of flasks at each pH were used as controls to ensure that the adjustment procedure itself had no effect on algal growth. These flasks were identical in all ways except that the pH was not adjusted but simply recorded daily using a pH meter.

2.5 Toxicity testing of selected metals

2.5.1 Experimental design

The general toxicity test design consisted of triplicate controls together with 7–10 metal concentrations, each with two to three flasks per treatment. A range-finding test was initially carried out using a wide range of metal concentrations. The concentrations chosen for the definitive tests were based on the EC_{50} values determined from the range-finding test. Concentrations were chosen with the aim of producing no effect at the lowest concentration and 100% effect at the highest concentration, and a range of partial effects at the intermediate concentrations. To ensure reproducibility of the test, three definitive toxicity tests were performed.

2.5.2 Toxicity test procedure

The standard toxicity test procedure used for metal testing was the same as that described in section 2.3, except for the following variations:

1 54.5 mL of synthetic softwater was added to the test flasks rather than 50 mL, giving a initial volume of 55 mL after addition of nutrients. This extra volume was necessary to allow for a 5 mL sub-sample to be taken for chemical analysis on day 0 (see below);

2 Prior to incubation, the pH of all flasks was adjusted to 5.7 ± 0.1 or 6.5 ± 0.1 by the addition of 0.02 M NaOH or 0.01 M HCl. Continued adjustment of the pH twice daily (morning and afternoon) was carried out throughout the duration of the test as part of the standard toxicity test procedure.

A 5 mg Cu L^{-1} and 100 mg Cu L^{-1} stock was prepared from copper sulphate ($CuSO_4 \cdot 5H_2O$) and acidified to 0.006 M and 0.012 M respectively, by the addition of HCl. Acidification of the stock solution was necessary to prevent adsorption of Cu to the container walls. Copper concentrations tested ranged from 1.25–640 µg Cu L^{-1}. Care was taken to ensure that the addition of Cu to the test flasks did not alter the pH and appropriate amounts of 0.1 M NaOH were added where necessary.

For the U toxicity tests, a 10 mg U L^{-1} was prepared from uranyl sulphate ($UO_2SO_4 \cdot 3H_2O$). Concentrations tested ranged from 5–320 µg U L^{-1}. No neutralisation step for U was necessary.

Immediately following the addition of Cu or U to the test flasks, 5 mL sub-samples were taken from each control and test flask for metal analysis. Copper samples were analysed by graphite furnace atomic absorbance spectrometry (GFAAS, Model 4100ZL). Uranium samples were analysed by inductively coupled plasma atomic emission spectrometry (ICPAES, Labtam Model 8410). Sub-samples from duplicate or triplicate flasks at each concentration were combined to give a total volume of 10–15 mL, which were then acidified

with 30 µL concentrated HNO_3 (Normatom). Nine samples were randomly selected from the complete set of toxicity tests and analysed for Na, K, Ca, Mg, and Cl by ICPAES. Six sub-samples were also randomly selected and analysed for nitrate and phosphate by the same method (not acidified). Concentrations of Al, Fe and Mn in the synthetic water were measured by ICPAES. All samples were refrigerated at 4°C prior to analysis.

2.6 The influence pH on metal toxicity

A series of algal growth inhibition tests were conducted at pH 6.5 to determine the effect of H^+ concentration on the toxicity of Cu and U to *Chlorella* sp. The toxicity tests were conducted in the same manner as outlined above for metal testing at pH 5.7 (section 2.5) except that the pH of the test medium was pre-adjusted to pH 6.5 ± 0.1.

Data were analysed using statistical procedures outlined in Section 2.3.5. The effect of H^+ concentration on the toxicity of Cu and U to *Chlorella* sp was determined by comparing EC_{50} values at pH 5.7 and 6.5.

2.7 Intracellular and extracellular metal determination

To investigate the mechanism by which pH may influence metal toxicity, a quantitative experiment was undertaken to determine intracellular and extracellular (membrane associated) metal concentrations (Cu and U) after 72 h exposure.

Copper experiment

Flasks (four replicates) were set up at pH 5.7 and pH 6.5 using the test procedure outlined in section 2.5.2. Three Cu concentrations were prepared at each pH (pH 5.7 = 10, 80 and 640 µg Cu L^{-1}) (pH 6.5 = 10, 80 and 160 µg Cu L^{-1}), in order to cover a range of Cu toxicity values. One flask from each treatment was used to determine cell density daily for three days. These flasks acted as a test control to allow a comparison with earlier toxicity tests. The remaining three flasks per treatment were counted only on day 3 to allow sufficient volume for intracellular and extracellular Cu determination. All flasks were pH adjusted twice daily as part of the standard test protocol.

Uranium experiment

Determination of intracellular and extracellular U was carried out in the same manner as that described for Cu at three U concentrations for both pH 5.7 and pH 6.5 (40, 120 and 250 µg U L^{-1}).

2.7.1 Experimental procedure

At the completion of the toxicity test (day 3), the remaining test medium was retained. In a class 100 clean room, 40 g of solution from each flask was decanted into 50 mL plastic centrifuge tubes which had been presoaked overnight in 50% HNO_3 and thoroughly washed with Milli-Q water. Samples were centrifuged at 3500 rpm at 20°C in a Joun CR4.11 refrigerated centrifuge for 20 min. Twenty mL of the solution (supernatant) was pipetted into clean acid-washed (10% HNO_3) polycarbonate vials and acidified with 40 µL concentrate HNO_3. These samples were analysed for dissolved Cu by GFAAS and dissolved U by ICPAES.

A further 10 mL of the supernatant was discarded and the remaining 10 mL re-centrifuged for 5 min. This additional centrifugation step was necessary so that the cells formed a distinct pellet. Following this, the remaining supernatant was discarded, with the exception of approximately 0.70–0.75 mL that was retained in the tubes so as not to disturb the algal pellet.

The algal pellet was re-suspended in 20 mL of 0.02 M EDTA and shaken for approximately 30 s. This EDTA removed the Cu or U bound to the outside of the cells (Florence & Stauber 1986). The samples were then centrifuged for a further 20 min at 4000 rpm and the supernatant removed and retained for Cu or U analysis. No acidification was necessary. These samples were referred to as the 'extracellular Cu or U' fraction.

The algae were left to dry in a laminar flow hood for two days and then acid digested with 2 mL concentrate HNO_3. Samples were left for 30 min and then microwaved on a power setting of 1 for 5 min. After cooling to room temperature, the samples were made up to 20 mL with Milli-Q water and analysed for Cu by GFAAS and U by ICPAES. These samples were referred to as the 'intracellular Cu or U' fraction.

In order to account for any Cu or U that may have adsorbed to the walls of the glass flask over the duration of the test, the emptied flasks were filled with 50 mL of 0.03 M HNO_3 and left overnight. Copper was analysed by GFAAS and U by ICPAES and these were referred to as the 'flask bound Cu or U' fraction.

2.8 Toxicity test with *Chlorella* sp 12 (Papua New Guinea)

For comparison with the Kakadu isolate of tropical *Chlorella* a toxicity test was also performed using a *Chlorella* isolate from Lake Aesake, Papua New Guinea (*Chlorella* sp 12).

The species was maintained at the laboratories of the CSIRO Division of Energy Technology, Sydney. The alga was cultured axenically in 1/5 strength Jaworki's medium (Thompson et al 1988) and maintained on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 120 µmol photons PAR m^{-2} s^{-1}) at 27 ± 1°C.

A growth inhibition test with this species was performed according to the standard protocol used at the CSIRO Division of Energy Technology, Sydney (Stauber unpublished data). The test medium consisted of a synthetic softwater having a water hardness 80–90 mg L^{-1} as $CaCO_3$ ($NaCO_3$, 96 mg L^{-1}; $CaSO_4 \cdot 2H_2O$, 60 mg L^{-1}; $MgSO_4 \cdot 7H_2O$, 60 mg L^{-1}; KCl, 4 mg L^{-1}). Triplicate controls, together with six Cu concentrations, ranging from 2.5–40 µg L^{-1} (each in triplicate) were prepared in clean silanised 250 mL Erlenmeyer flasks containing 50 mL of synthetic softwater. The addition of nutrients, algal cell counts and other test procedures were as described in section 2.3 for *Chlorella* sp (Kakadu isolate). Statistical analyses were the same as that described in section 2.3.5.

2.9 The influence of HEPES buffer on copper and uranium toxicity

A series of Cu and U toxicity tests were performed to investigate the effect of adding HEPES buffer (*N*-2 hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) to the medium to maintain a constant pH over the 72 h test period. The buffer was prepared as a 0.5 M stock solution. The test consisted of an unbuffered control media (standard synthetic softwater) and two test treatments with 2 mM and 5 mM HEPES buffer, respectively. The toxicity tests were performed in the same manner as outlined in section 2.5.

3 Algal toxicity test and instrument optimisation

3.1 Instrument optimisation: Coulter calibration

Due to the often imprecise and time-consuming nature of manual cell enumeration techniques, automation of cell counts was investigated using a Coulter Multisizer II particle analyser. In this technique, algal cells are suspended in an electrolyte (seawater). As they are passed

through a small aperture, across which an electric current has been established, each cell causes a voltage drop which is recorded as a count. The height of the pulse is proportional to the volume (size) of the cell, and as such, the Coulter Counter gives an estimate of cell size distribution as well as cell numbers (Stauber et al 1994).

Prior to using the Coulter Counter as part of the standard counting technique, a calibration experiment was performed to determine whether the Coulter Counter would be suitable for the range of cell densities expected in the toxicity tests. This was tested with a series of dilutions of *Chlorella* in seawater from 1:5 to 1:200 (fig 2). The Coulter counts (actual counts) agreed well with the theoretical counts (determined by counting the highest cell concentration and calculating the expected cell number at each dilution) over all dilutions with a correlation coefficient (r^2) = 0.97. However, at very low cell counts (<4500 cells/0.1 mL), the instrument was less accurate at determining cell density as the background particle counts and algal cell counts were often indistinguishable.

To overcome this problem, determination of the algal inoculum for day 0 was performed using the microscope. At very high cell counts (>70 000 cells/0.1 mL), the Coulter Counter tended to underestimate cell density, a common problem with this instrument as it counts two cells as one. To correct for this, the instrument has an automatic coincidence correction (%). Provided the coincidence correction was <35%, and the cell count >4500, excellent correlation between actual and theoretical counts was found (r^2 = 0.99). In all algal toxicity tests, samples were diluted to keep cell counts below 70000 cells/0.1 mL and the coincidence correction <35%.

To compare both manual counts using a microscope and automatic Coulter counts, *Chlorella* cells were counted by both methods for a number of toxicity tests and at a range of dilutions in seawater. Figure 3 shows Coulter counts plotted against microscope counts with a calculated linear regression line. There was excellent agreement between cell numbers determined by both microscope and Coulter counting techniques, with a correlation coefficient of r^2 = 0.98. The Coulter Counter has several advantages over the manual microscope counts, including its ability to count thousands of cells, high precision, speed and the absence of field errors inherent in haemocytometer counting. The Coulter Counter was subsequently used to count cells in all algal toxicity tests except on day 0 when cell densities were low.

3.2 Determination of algal inoculum age and size

The growth of the tropical freshwater alga *Chlorella* sp over 21 d in a high nutrient culture medium is shown in figure 4. Cells grew exponentially for up to 4–5 d with no lag phase evident. After this time, a reduction in growth rate occurred as the nutrients in the medium became depleted. Cells then entered the stationary phase and maintained stationary growth (cell division rate = cell death rate) for at least 15 d.

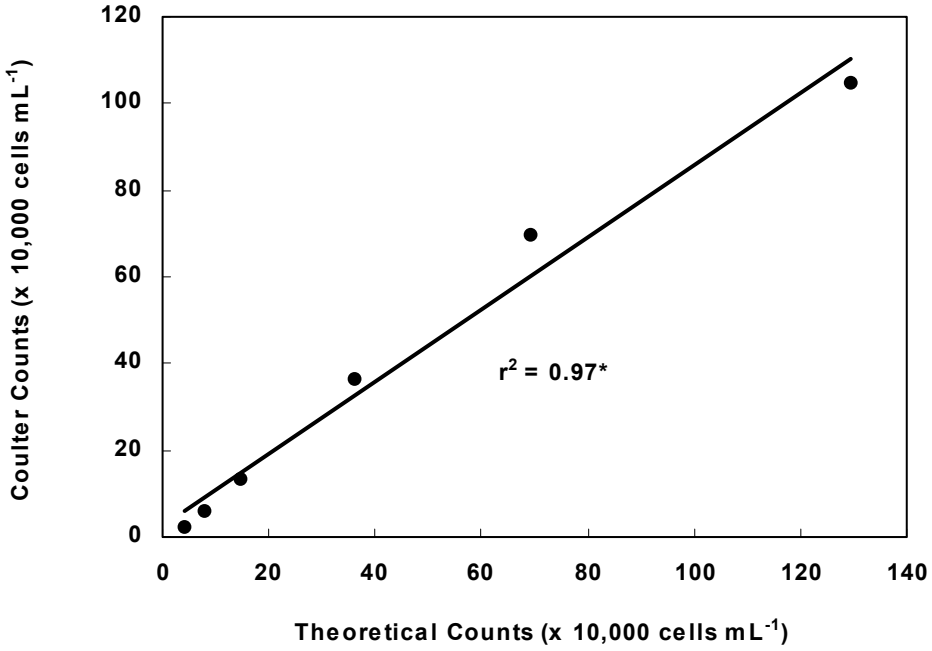


Figure 2 Growth of *Chlorella* sp – actual Coulter count v theoretical Coulter count (calculated from the cell dilution) * ($P \leq 0.05$)

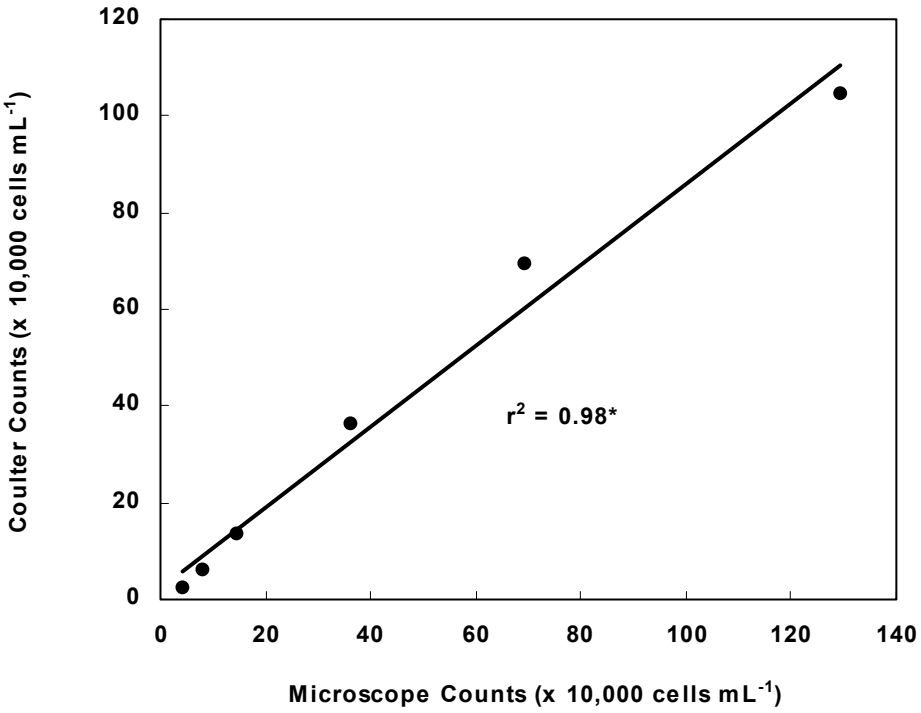


Figure 3 Growth of *Chlorella* sp – actual Coulter count v microscope Coulter count * ($P \leq 0.05$)

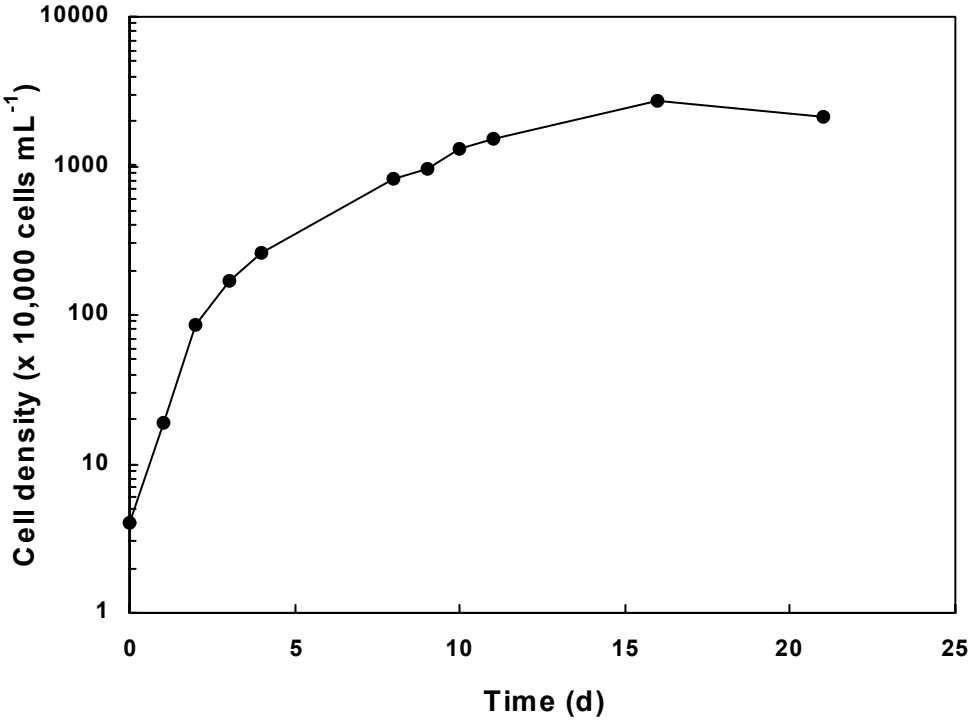


Figure 4 Growth of *Chlorella* sp in MBL medium over 21 d (all SD <500 cells mL^{-1})

As part of all standard toxicity test protocols, exponentially growing cells are required for toxicity testing in order to reduce the lag period at the beginning of the test (Stauber et al 1994). Four to five day old inocula were therefore used in the standard test protocol for *Chlorella* sp.

The number of cells initially added to the test flasks (the test inoculum) has been shown to influence the toxicity of certain metals (Pascucci & Sneddon 1993). In general, the higher the initial cell density, the less sensitive the algae are to the toxicant. The initial cell density was therefore standardised to $2–4 \times 10^4$ cells mL^{-1}, as this has been shown for other species to be low enough for maximum sensitivity of the test, and high enough to obtain good cell densities for counting over a 72 h period (Stauber et al 1994). Similar initial cell densities have been used in standard USEPA freshwater toxicity tests (USEPA 1994).

3.3 Optimisation of algal toxicity test parameters

3.3.1 Nutrients

Stauber and Florence (1989) have demonstrated that algal culture media, typically very high in nutrients and containing chelators and adsorbents, can ameliorate the toxicity of some metals. To overcome this problem, protocols for toxicity testing with freshwater species have been developed and recommend the use of synthetic water, enriched only in nitrate and phosphate (Stauber et al 1994). A series of experiments were carried out to determine the optimum nutrient conditions for growth of *Chlorella* sp without significantly affecting the toxicity of metals. Control cell division rates of *Chlorella* sp at varying nitrate and phosphate concentrations in the test softwater are shown in figures 5 and 6. Summary data are provided in appendix C. The pH of the test medium for the duration of the toxicity test ranged from pH 6.4 (day 0) to pH 7.4 (day 3).

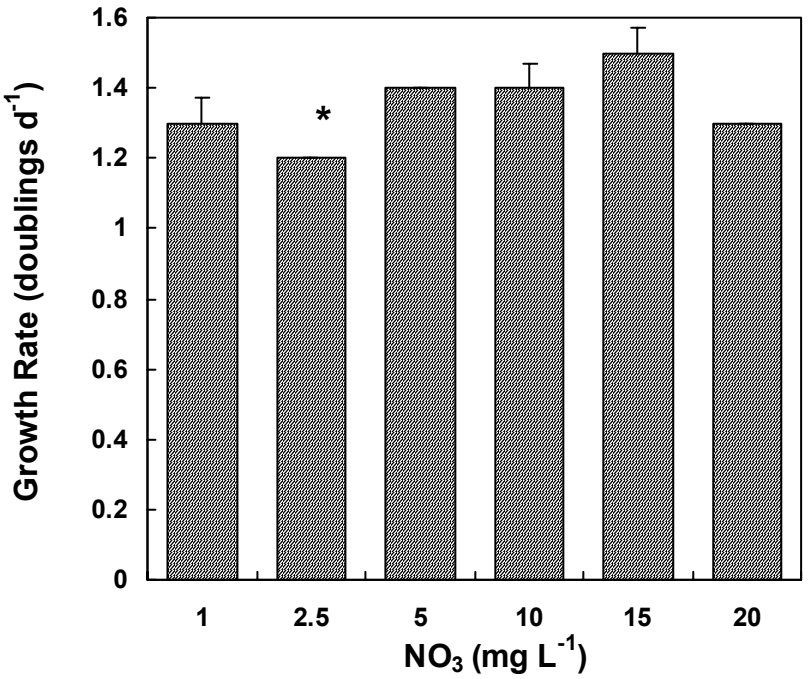


Figure 5 Mean control cell division rates of *Chlorella* sp at varying nitrate concentrations. Error bars represent one SD. * Indicates significant (P ≤0.05) difference from the 15 mg L^{-1} treatment.

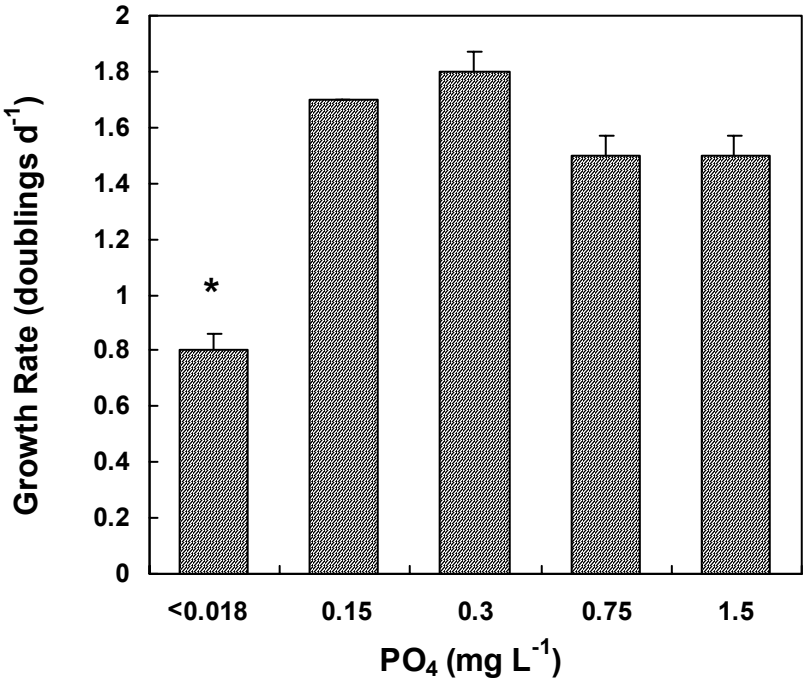


Figure 6 Mean control cell division rates of *Chlorella* sp at varying phosphate concentrations. Error bars represent one SD. * Indicates significant (P ≤0.05) difference from the 0.15 mg L^{-1} treatment.

At the range of nitrate concentrations tested, a maximum cell division rate of 1.53 doublings d^{-1} was obtained with the addition of 15 mg NO_3 L^{-1}. The growth rate at this nitrate concentration was not significantly ($P > 0.05$) different to those of all other treatments, with the exception of that at 2.5 mg NO_3 L^{-1}. Cells, however, ceased to grow exponentially over the 3 d test at nitrate concentrations of <5 mg NO_3 L^{-1}. Synthetic softwater, enriched with 15 mg NO_3 L^{-1}, was therefore chosen for the standard test protocol. This nitrate concentration is consistent with the standard concentration used for 72 h algal toxicity tests with Australian temperate species.

Chlorella cells did not grow well without the addition of phosphate to the medium. At the background concentration of phosphate (<18 µg PO_4 L^{-1}, based on Magela Creek water), control cell division rates were less than 1 doubling d^{-1}, and a lag phase of about one day was observed before the onset of growth.

Good algal growth rates were obtained at all other phosphate concentrations tested (0.15–1.5 mg PO_4 L^{-1}) with continuous exponential growth for at least 3 d. A maximum cell division rate of 1.77 doublings d^{-1} was obtained with the addition of 0.3 mg PO_4 L^{-1}. This was not significantly different from all other phosphate concentrations tested except the background level. Because phosphate is known to complex strongly with metals, particularly U, it was important to keep the phosphate concentration in the test medium as low as possible. Since *Chlorella* sp could not grow acceptably without the addition of phosphate, synthetic softwater enriched with only 0.15 mg PO_4 L^{-1} was chosen for the standard test protocol as it offered least possible interference with metal toxicity. As with nitrate, this phosphate concentration is consistent with other standard 72 h algal toxicity tests in Australia (Stauber et al 1994). The pH of the test medium for the duration of the toxicity test ranged from pH 6.2 (day 0) to pH 7.0 (day 3).

3.3.2 Light intensity

Control cell division rates of *Chlorella* sp at three different light intensities are shown in table 5. Cells responded best to the highest light intensity of 115–125 µmol photons PAR $m^{-2}s^{-1}$, reaching 1.40 doublings d^{-1}. Although algal growth was greatest at this light intensity, it was not significantly ($P > 0.05$) different from the lower light intensities. The highest light intensity was chosen for the standard toxicity test. The pH of the test medium for the duration of the test ranged from pH 6.3 (day 0) to pH 6.9 (day 3).

Table 5 Control cell division rates of *Chlorella* sp at varying light intensities

Light intensity (µmol photons PAR $m^{-2}s^{-1}$)	Mean cell division rate (doublings d^{-1})	pH	
		Day 0	Day 3
32–38	1.16 ± 0.1	6.4	6.5
70–72	1.35 ± 0.1	6.4	6.9
115–125	1.40 ± 0.1	6.3	6.9

3.3.3 Gas exchange

The effect of continuous shaking at 100 rpm on the growth rate of the alga over 72 h was compared to shaking the test mini-vials twice daily by hand (ie non-shaking) (table 6). Under both shaking and non-shaking incubation conditions, control cell division rates in the mini-vials were about 1.40 ± 0.1 doublings d^{-1}, indicating that the effect of shaking on control algal growth was negligible. Control cell division rates in the standard Erlenmeyer flasks (non-

shaking) were 1.16 ± 0.1 doublings d^{-1}, resulting in no significant ($P > 0.05$) difference between the flask and mini-vial test methods. However, the pH of the medium in the mini-vials for the duration of the toxicity test rose from pH 6.1 (day 0) to pH 8.9 (day 3). pH control was much better in the non-shaking flasks, with the pH maintained at 6.4–6.5 over the three days. Manual shaking of the test flasks twice daily (USEPA 1994) was chosen as the best method to promote gas exchange and to maintain pH control over the three days.

Table 6 Control cell division rates of *Chlorella* sp under shaking and non-shaking incubation conditions

Incubation condition (shaking/non-shaking)	Mean cell division rate (doublings d^{-1})	pH	
		Day 0	Day 3
shaking (mini-vial)	1.40 ± 0.1	6.1	8.9
non-shaking (mini-vial)	1.45 ± 0.1	6.6	8.5
non-shaking (flask)	1.16 ± 0.1	6.4	6.5

3.3.4 pH

An on-going concern in metal toxicity tests is the control of pH which can influence the speciation, and hence, toxicity of a metal (Meador 1991, Markich et al 1996). As a result of the utilisation of carbon dioxide and bicarbonate, and the uptake of nitrogen by algal cells, the pH of the test medium typically increases over the duration of the toxicity test (Lage et al 1996). Results of previous optimisation tests had shown large increases in pH of >1.0 pH unit over 72 h.

To overcome the problem of increasing pH in the flasks over the test period a number of standard algal toxicity tests have used pH buffers, such as HEPES, to maintain pH at required levels (Stauber & Florence 1989, Stauber et al 1994). While effective in controlling pH (Stauber et al 1994, USEPA 1994), buffers have been shown to complex metals (L Hales pers comm) and also alter metal toxicity (Lage et al 1996). As a consequence, it has generally been regarded that the use of buffers in metal toxicity tests should be avoided.

An alternative method of pH control is the daily addition of HCl or NaOH to the unbuffered synthetic softwater. This method was investigated at pH 5.7 and pH 6.0. While initial optimisation tests confirmed that this species grew well at pH 6.0, a slightly lower pH value was required to cover the range of conditions typical of Australian tropical freshwaters.

Cell division rates of *Chlorella* sp at pH 5.7 and 6.0, and the effect of pH adjustment on algal growth, are shown in table 7.

Table 7 Control cell division rates of *Chlorella* sp at pH 5.7 and 6.0 and the effect of pH adjustment

pH	Mean cell division rate (doublings d^{-1})
5.7 (unadjusted)	1.45 ± 0.1
5.7 (adjusted)	1.44 ± 0.1
6.0 (unadjusted)	1.61 ± 0.1
6.0 (adjusted)	1.52 ± 0.1

Chlorella sp grew well at pH 5.7, with cell division rates in the pH-adjusted and pH-unadjusted treatments of approximately 1.4 doublings d^{-1}. Although algal growth was significantly ($P \leq 0.05$) lower at pH 5.7 compared to pH 6.0, cell division rates were comparable to those used in standard test protocols for freshwater unicellular algae (Stauber et al 1994). Any change to algal cell division rates due to the pH adjustment process was negligible at both pH values.

This alternative method of pH control was effective in maintaining the pH close to the required levels from days 0 to 2 of the toxicity test (appendix C). Over this period, less than 0.5 pH unit increase was observed at both pH 5.7 and pH 6.0. By day 3, however, pH in the synthetic softwater controls rose by about 1.0 pH unit. This increase in pH was due to utilisation of carbon dioxide and nitrogen by increasing algal cell densities, particularly in controls containing high cell densities by day 3. However, as metal speciation and other related metal properties vary with pH, it is essential that large increases in pH be avoided. The pH of the medium was therefore subsequently adjusted twice daily (morning and afternoon) in an attempt to overcome this problem.

3.3.5 Other test parameters and summary of initial toxicity test protocol

A temperature of 27 ± 1°C was chosen for good algal growth of *Chlorella* sp. This temperature is the median surface water temperature in Magela Creek, Northern Territory, and is considered typical of the region over the wet season (Markich & Camilleri 1997). This temperature is also consistent with the majority of toxicity studies using tropical Australian species (Markich & Camilleri 1997).

A summary of the initial toxicity test protocol for *Chlorella* sp is shown in table 8. The complete test protocol is detailed in appendix A. Included in the protocol are the results of those parameters tested in the optimisation experiments, as well as test parameters that have been standardised from other algal toxicity tests, including light quality and test volume.

Table 8 Summary of initial toxicity test protocol for the tropical *Chlorella* sp growth inhibition test

1.	Test type	Static
2.	Temperature	27 ± 1°C
3.	Light quality	Cool white fluorescent lighting
4.	Light intensity	115–125 µmol photons PAR $m^{-2}s^{-1}$
5.	Photoperiod	12 h light: 12 h dark
6.	Test chamber size	250 mL
7.	Test solution volume	50 mL
8.	Renewal of test solutions	None
9.	Age of test organisms	4–5 d
10.	Initial cell density in test chambers	$2–4 \times 10^4$ cells mL^{-1}
11.	No. of replicate chambers/concentration	2–3
12.	Shaking rate	Twice daily by hand
13.	Test medium	Synthetic softwater + 15 mg L^{-1} NO_3 + 0.15 mg L^{-1} PO_4 (pH 5.7)
14.	Metal concentrations	Minimum of 5 and a control
15.	Test duration	72 h
16.	Test endpoint	Growth (cell division)
17.	Test acceptability	Control cell division rate 1.40 ± 0.3 doublings d^{-1}; coefficient of variation in controls <20%

To help ensure reproducibility of the toxicity test, a test acceptability criterion was calculated from the optimisation tests, giving a mean cell division rate of 1.40 ± 0.3 doublings d^{-1}. Control cell division rates which did not fall within this range of acceptability, or that had a coefficient of variation (CV) of >20% between replicates, were not subsequently used in deriving toxicity results. Similar control cell division rates (1.3 ± 0.3 doublings d^{-1}) have been reported for *Chlorella* sp 12 (PNG isolate) and *Chlorella protothecoides* (Stauber et al 1994).

3.4 Discussion

Using the tropical freshwater alga, *Chlorella* sp isolated from Kakadu National Park, Northern Territory, a standard toxicity test protocol was successfully developed using suitable incubation conditions, inoculum age and test media. As is now recommended for toxicity testing with algae (Stauber 1995), the composition of the test medium should resemble, as closely as possible, the natural water chemistry of the system. Magela Creek, typical of the sandy braided streams in tropical northern Australia, is characteristically very soft, slightly acidic and has a low buffering and complexing capacity (Hart et al 1987). Consequently, the toxicity of metals to aquatic organisms under these conditions is likely to be higher than in natural waters rich in organics.

Most algal toxicity tests used overseas (Environment Canada 1992, USEPA 1994) are carried out in high-nutrient culture media. Typically, these media contain nutrients, such as phosphate, silicate, manganese and iron, at concentrations that greatly exceed those in natural waters. Because these media components can complex and adsorb metals, the toxic effect is often severely underestimated (Stauber & Florence 1989). The newly developed test protocol for *Chlorella* sp overcomes this problem by using a synthetic softwater supplemented with minimal nutrients (nitrate and phosphate only), and avoids the use of complexing agents, such as EDTA, which have a strong affinity for metal ions (Wong et al 1995). The use of a standard softwater medium also provides a baseline from which a large range of different water quality parameters can be calibrated and assessed against natural waters (Markich & Camilleri 1997).

4 Toxicity of copper to *Chlorella* sp

4.1 Significance of copper toxicity testing in tropical Australia

Copper is known to be one of the more toxic metals to freshwater biota, and as such, is commonly the metal of most concern in aquatic systems affected by metal pollution (Harrison & Bishop 1984, Nor 1987). To protect freshwater ecosystems, the 1992 ANZECC water quality guidelines for total Cu range from 1 to 5 $\mu g\ L^{-1}$ (depending on water hardness).

Unfortunately, no quantitative formula is provided in the guidelines to specify a particular Cu concentration for a particular hardness.

In this study Cu was selected as a priority metal for toxicity testing due to its potential ecotoxicological impact in northern Australia where mining is a major activity. Although local data on the toxicity of Cu to tropical animals are gradually increasing (Markich & Camilleri 1997), no information on the toxicity of Cu to local species of freshwater algae is currently available.

4.2 Copper toxicity to *Chlorella* sp at pH 5.7

The toxicity of Cu to the new tropical isolate *Chlorella* sp was initially determined in a range-finding test using nominal Cu concentrations of 2.5–160 µg Cu L^{-1} (data not presented). To ensure algal growth inhibition to Cu responded in a reproducible way, three definitive toxicity tests were then performed. Based on the results of the range-finding test, additional nominal concentrations of 1.5, 320 and 640 µg Cu L^{-1} were added to the test concentrations in order to refine the concentration-response curve.

Summary data for three Cu toxicity tests are given in appendix D. Good control growth rates were obtained for all toxicity tests (~1.4 doublings d^{-1}) with the % CV of <20%, indicating test acceptability. Continuous exponential growth was maintained in all control treatments.

Measured Cu concentrations at the beginning of the toxicity tests agreed closely (typically 5–10% CV) with nominal concentrations. pH control in the unbuffered water was reasonable throughout the test and typically increased <0.5 pH unit over 72 h. In the control treatments, and on occasion the low Cu treatments, the pH increased to about pH 6.9 by the afternoon of day 2 before readjustment to pH 5.7.

Growth of the alga typically decreased with increasing Cu concentration. Three separate 72 h toxicity tests gave EC_{50} values of 20, 32 and 38 µg Cu L^{-1} (table 9). Since the 95% confidence intervals from each test overlapped, these values were not considered significantly different. Copper concentrations of 1.4 µg Cu L^{-1} did not significantly affect algal growth, with the lowest observable effect concentration at 2.3 µg Cu L^{-1}.

Table 9 Summary of test endpoints on Cu toxicity to *Chlorella* sp at pH 5.7

Test-run	Mean cell division rate (doublings d^{-1})	Cu toxicity (µg L^{-1})		
		72 h EC_{50} (95% CI)	NOEC	LOEC
1st definitive	1.46	20 (12–32)	<2.6	2.6
2nd definitive	1.34	32 (17–63)	<2.5	2.5
3rd definitive	1.29	38 (30–46)	1.4	2.3

Data from the three definitive tests were pooled and a combined concentration-response curve of the toxicity of Cu to *Chlorella* sp (at pH 5.7), together with calculated statistical endpoints, is shown in figure 7. At low concentrations of Cu (1.4 µg Cu L^{-1}) only minor reductions in algal growth occurred. As Cu concentrations increased, a gradual decrease in algal growth was observed over the concentration range 2.4–19 µg Cu L^{-1}, reducing growth to approximately 55% of the control. Further increases in Cu concentration had little effect (<10%) on algal growth rate, up until a threshold point of 300 µg Cu L^{-1} at which pronounced inhibition occurred. The 72 h EC_{50} value was 35 µg Cu L^{-1} with 95% confidence intervals of 28–42 µg Cu L^{-1}. This value is consistent with the mean 72 h EC_{50} value of 30 µg Cu L^{-1} (CI 20–57 µg Cu L^{-1}) derived from the individual test-runs (table 9).

The shape of the concentration-response curve for *Chlorella* sp (Kakadu isolate) does not represent the typical sigmoidal curve shown by most algal species in the presence of a toxicant. For comparative purposes, a toxicity test was performed using the closely related tropical *Chlorella* sp 12 (Papua New Guinea isolate) which is used by the CSIRO Division of Energy Technology as part of routine testing (Stauber unpublished data). Figure 8 shows the results of this toxicity test in which a typical sigmoidal response curve to Cu was found.

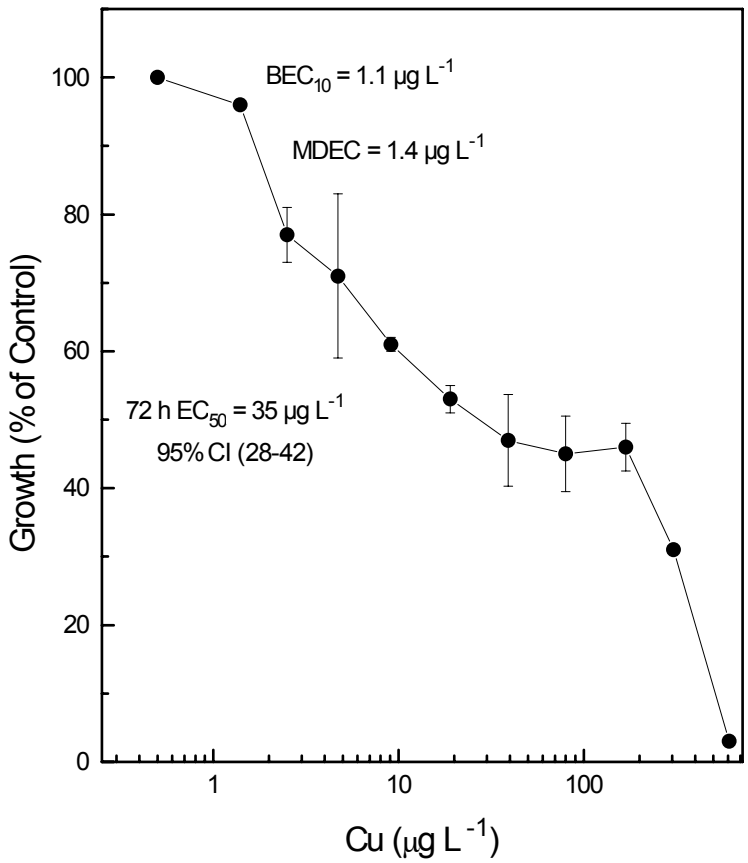


Figure 7 Growth inhibition of *Chlorella* sp exposed to Cu over 72 h at pH 5.7. Data points represent the mean of three tests ± one SD (except for 1.4, 306 and 611 µg Cu L^{-1}).

At low concentrations of the toxicant, little or no growth inhibition was detected. Above a certain threshold, small changes in the toxicant concentration typically caused pronounced growth inhibition. Complete growth inhibition then occurred as the toxicant concentration was further increased. The 72 h EC_{50} value was 8.4 µg Cu L^{-1}, with 95% confidence intervals of 8.0–8.8 µg Cu L^{-1}.

The advantage of a sigmoidal type response is that because the response curve is very steep at the intermediate concentration range, a much more reproducible EC_{50} value with narrow 95% confidence intervals is obtained (ie the median percentile growth response occurs around the same toxicant concentration).

The concentration-response curves obtained for the new tropical isolate, *Chlorella* sp (from Kakadu) showed significant deviation from this typical sigmoidal response. The initial drop in algal growth, followed by a flattening of the response curve, means that the 50% inhibition of growth (EC_{50}) was derived from a flatter part of the curve with broad 95% confidence intervals. An increase in Cu concentration from 20–160 µg Cu L^{-1} resulted in <10% change in growth inhibition. As such, small variations in the alga's response to Cu between toxicity tests, may result in substantial differences in the EC_{50} value obtained.

In an attempt to overcome the problem associated with the unusual concentration-response curves for this species, alternative test endpoints were investigated. Using 72 h toxicity data, the EC_{25} and EC_{75} values were derived using Maximum Likelihood-Probit analysis (Hamilton et al 1977). Because the concentration-response curves were steepest at the lower and higher Cu concentrations tested the EC_{25} and EC_{75} values should theoretically be more appropriate and

reproducible test endpoints. The EC_{25} value is increasingly being used by Environment Canada for toxicity assessment (Kovacs et al 1995). The 72 h EC_{25} value from the pooled data was 4.9 µg Cu L^{-1} with 95% confidence intervals of 1.3−11 µg Cu L^{-1}. This endpoint did not significantly differ between individual test-runs (ie overlapping 95% confidence intervals). At the other end of the concentration-response curve, the EC_{75} value was found to be an inappropriate test endpoint due to very large confidence intervals (appendix E).

Two additional statistical measures for assessing the toxicity of metals to *Chlorella* sp were also calculated; the 10% bounded effect concentration (BEC_{10}), and the minimum detectable effect concentration (MDEC). While the EC_{50} is a commonly used statistical endpoint in toxicity studies, the BEC_{10} and MDEC have received little attention. These values are alternative statistical measures to the no-observable effect concentration (NOEC) and lowest-observable effect concentration (LOEC) respectively (see section 2.3.5). NOEC and LOEC values are typically used in risk extrapolations (Aldenberg & Slob 1993) to derive acceptable levels of a pollutant which protects 95% of species in the ecosystem. Based on concentration-response data, rather than hypothesis testing (as used in deriving NOEC and LOEC values), the BEC_{10} and MDEC are thought to provide a more appropriate starting point for environmental risk analysis for a number of reasons, including:

- the procedure is well-defined for interpolation of effects to untested concentrations. In contrast, the NOEC and LOEC are always test concentrations and do not innately correspond to specific effects concentrations (Hoekstra & van Ewijk 1993).
- all the information in the concentration-response curve may be used in the analysis. This information is lost when using the hypothesis testing approach, and as such, the investigator has no means of evaluating the test results (Moore & Caux 1997).

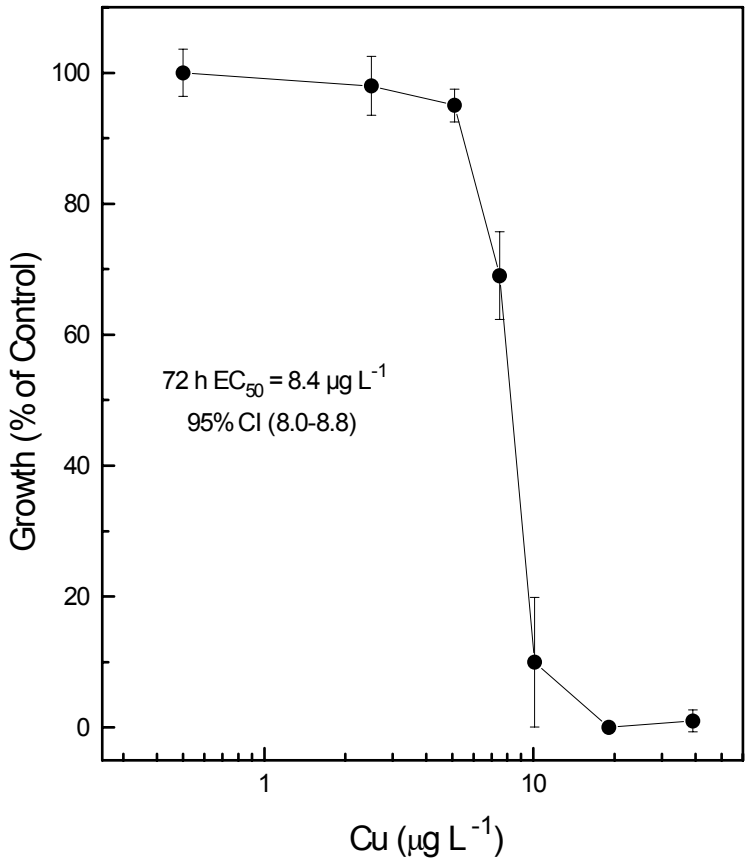


Figure 8 Growth inhibition of *Chlorella* sp 12 exposed to Cu over 72 h. Error bars represent one SD.

A more detailed discussion of the advantages of the concentration-response approach over hypothesis testing are given by Suter (1996) and Moore and Caux (1997).

As a result of the pronounced growth inhibition of *Chlorella* sp at low Cu concentrations, the calculated BEC_{10} and MDEC values were 1.1 µg Cu L^{-1} and 1.4 µg Cu L^{-1} respectively. These values were slightly lower than the NOEC and LOEC values calculated by the hypothesis testing approach (table 10). This indicates that the concentration-response approach is a more sensitive measure for detecting subtle disturbances caused by the presence of Cu.

The usefulness of the 48 h data in assessing the toxicity of Cu to *Chlorella* sp was also investigated. The obvious advantage of conducting 48 h toxicity tests would be the reduced experimental time and effort needed. Moreover, a shorter duration would also help ensure adequate pH control of the test medium. The data were subsequently re-analysed for each toxicity test over this period and a 48 h EC_{50} value generated (appendix E). These values were typically higher than those derived from 72 h data (ie less sensitive) and were less reproducibile between individual test-runs. As such, the 72 h EC_{50} value was found to give more reliable and reproducible results.

4.3 The influence of pH on copper toxicity

Metal toxicity to algae is highly pH-dependent. Many studies have revealed marked decreases in metal toxicity at lower pH (Aliotta et al 1983, Peterson et al 1984); however, several researchers have reported the opposite effect (Starodub et al 1987, Rai et al 1993). This inconsistency has meant that conclusive metal-pH effects have not been firmly established. Hence, one objective of the present study was to investigate the influence of pH on the toxicity of Cu to *Chlorella* sp and to compare cellular Cu uptake at two different pH values (pH 5.7 and 6.5).

4.3.1 Comparison of control algal growth rates at varying pH

In order to determine an appropriate level at which to test the effect of pH on Cu toxicity, control cell division rates of *Chlorella* sp were compared over a range of pH values measured in Australian tropical freshwaters. Figure 9 shows control growth rates of *Chlorella* sp at pH 5.0, 5.7, 6.5 and 7.0 over 72 h. This species grew well at all pH values tested, with >1.0 doubling d^{-1}. A lag phase of about one day was observed at pH 5.0 and 7.0 before the onset of growth. A coefficient of variation of >20% was detected at pH 5.0. No apparent lag phase was evident at pH 5.7 and 6.5. A one-way analysis of variance showed that cells grew significantly ($P \leq 0.05$) better at pH 6.5 than at pH 5.7. No significant ($P > 0.05$) difference in algal growth rate was detected between pH 5.0 and 5.7 and also between pH 6.5 and 7.0. Because of the high variability found at pH 5.0, and the apparent lag phase observed at pH 5.0 and pH 7.0, a pH value of 6.5 was chosen, and a series of growth inhibition tests performed following the protocol outlined in section 2.3. Control of the test solution was reasonable for all pH treatments from days 0–2 of the test, however, an increase of >0.5 pH unit was observed by day 3 (appendix C).

4.3.2 Toxicity of Cu to *Chlorella* sp at pH 6.5

A range-finding test was not performed at pH 6.5. Concentrations tested were based on those used in the toxicity tests at pH 5.7, covering a range from 1.2–160 µg Cu L^{-1}. Summary data for each toxicity test are given in appendix D. High cell division rates of about 1.70 doublings d^{-1} were obtained in all controls with coefficients of variation of <20%, indicating test

acceptability. *Chlorella* sp was extremely sensitive to Cu at pH 6.5. Three separate 72 h toxicity tests gave consistent EC_{50} values, ranging between 1.1 and 3.6 µg Cu L^{-1} (table 10).

Table 10 Summary of test endpoints on Cu toxicity to *Chlorella* sp at pH 6.5

Test-run	Mean cell division rate (doublings d^{-1})	Cu toxicity (µg L^{-1})		
		72 h EC_{50} (95% CI)	NOEC	LOEC
1st definitive	1.72	3.6 (0.1–10)	<2.3	2.3
2nd definitive	1.67	1.1 (0.1–2.5)	<2.5	2.5*
3rd definitive	1.71	1.4 (1.0–1.9)	<1.2	1.2

* The LOEC value was higher than the EC_{50} therefore it has no ecotoxicological meaning in the context of the EC_{50} (values were derived using two separate techniques)

Measured Cu concentrations at the beginning of the toxicity tests agreed closely with nominal concentrations (typically <10% deviation). pH control in the unbuffered water was reasonable throughout the toxicity tests, rising by <0.5 of a pH unit over 72 h. The pH in control treatments, however, rose to pH 8.4 by day 2 of the test as a consequence of the high cell densities.

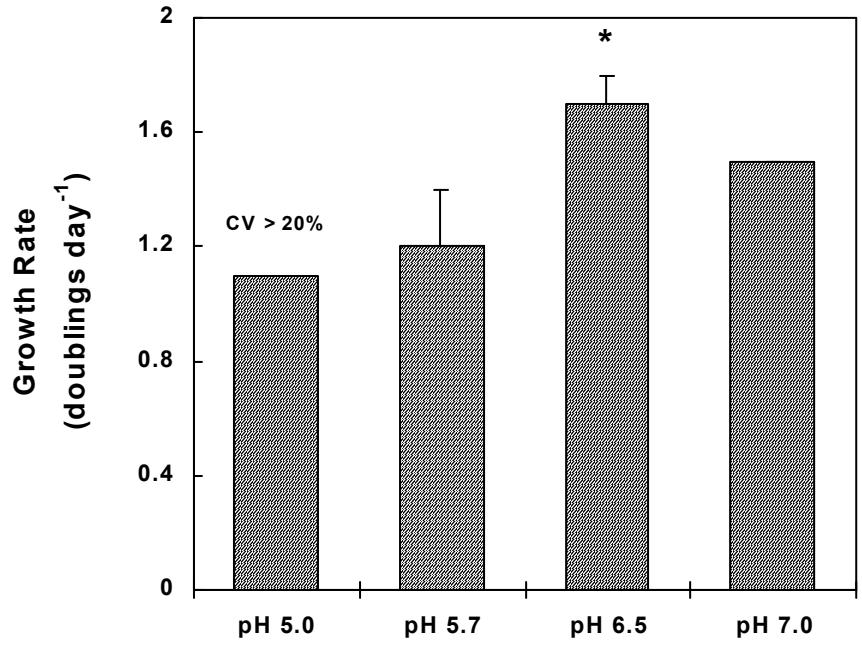


Figure 9 Growth of *Chlorella* sp in synthetic softwater at varying pH.
* indicates significant difference from pH 5.7 ($P \leq 0.05$).

A combined concentration-response curve of the toxicity of Cu to *Chlorella* sp at pH 6.5, together with calculated toxicological endpoints, is shown in figure 10. The concentration-response curve was initially very steep with pronounced growth inhibition at low Cu concentrations. BEC_{10} and MDEC values were 0.6 µg Cu L^{-1} and 0.7 µg Cu L^{-1} respectively. These values were close to background levels of Cu in the synthetic water (table 4). The concentration-response curve was quite flat over Cu concentrations from 1.2–80 µg Cu L^{-1}.

The 72 h EC_{50} value was 1.5 µg Cu L^{-1} with 95% confidence intervals of 0.8–2.8 µg Cu L^{-1}. Since this value was very low, an estimate of the EC_{25} value was not made.

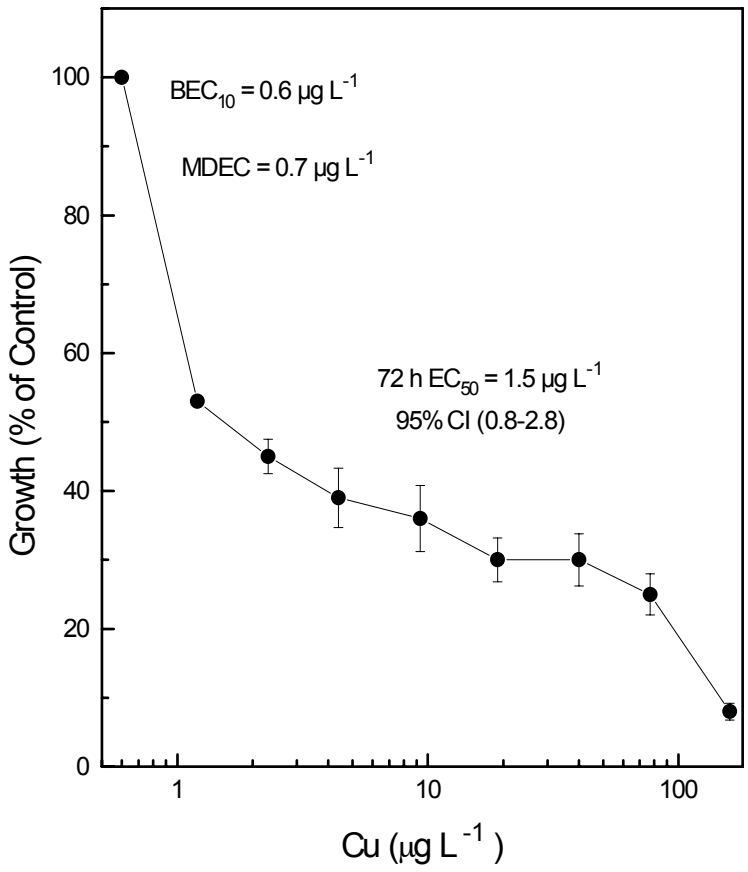


Figure 10 Growth inhibition of *Chlorella* sp exposed to Cu over 72 h at pH 6.5. Data points represent the mean of three tests ± one SD (except for 1.2 and 160 µg Cu L^{-1}).

Because good control growth rates (1.50 doublings d^{-1}) were obtained over 48 h, test endpoints were calculated over this period (appendix E). A similar shaped concentration-response curve was obtained (fig 11). The 48 h EC_{50} value showed good reproducibility between individual test runs, giving a combined value of 8.4 µg Cu L^{-1} with 95% confidence intervals of 4.4–16 µg Cu L^{-1}. The BEC_{10} and MDEC values of 0.6 µg Cu L^{-1} and 0.8 µg Cu L^{-1} respectively, were similar to those obtained over 72 h. The 48 h test had the added advantage of reducing experimental time and avoiding the pH control problems encountered over the last 24 h of the toxicity test.

4.3.3 Comparison of Cu toxicity to *Chlorella* sp at pH 5.7 and pH 6.5

It is clear from the results that Cu is significantly ($P \leq 0.05$) less toxic to *Chlorella* sp at pH 5.7 than at pH 6.5. Increasing the pH of the test medium resulted in a substantial reduction in the 72 h EC_{50} value from 35 µg Cu L^{-1} to 1.5 µg Cu L^{-1} (table 11). This corresponds to an 18-fold increase in Cu toxicity. However, based on the BEC_{10} and MDEC values, only a two-fold increase in Cu toxicity was observed as the pH increased from 5.7 to 6.5.

Table 11 Comparison of test endpoints for *Chlorella* sp at pH 5.7 and pH 6.5

	Cu toxicity (µg L^{-1})	
	pH 5.7	**pH 6.5**
72 h EC_{50} (95% CI)	35 (28–42)	1.5 (0.8–2.8)
BEC_{10}	1.1	0.6

MDEC	1.4	0.7

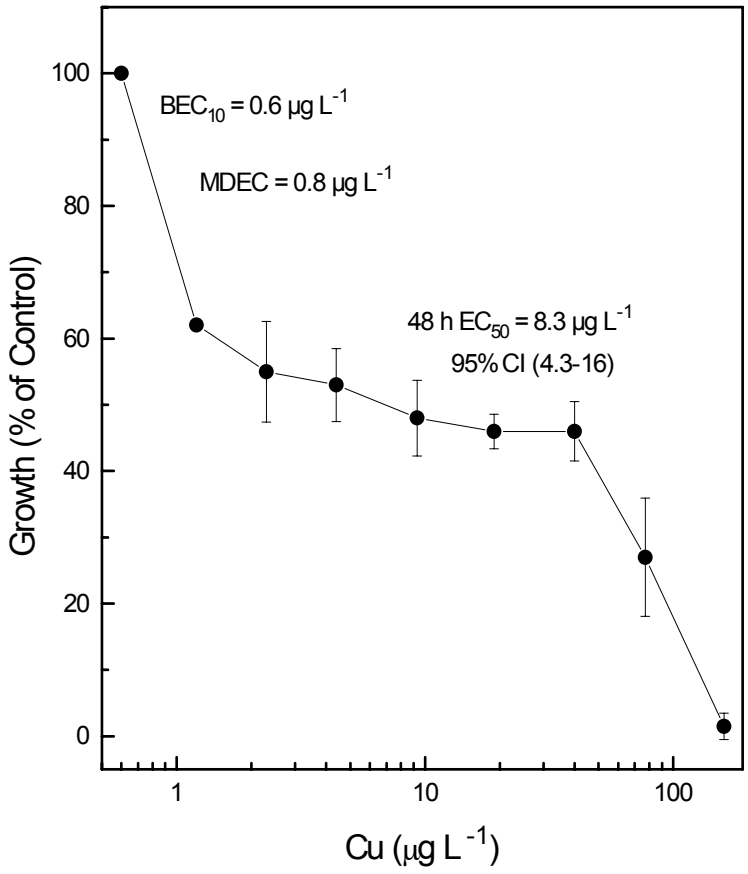


Figure 11 Growth inhibition of *Chlorella* sp exposed to Cu over 48 h at pH 6.5. Data points represent the mean of three tests ± one SD (except for 1.2 and 160 µg Cu L^{-1}).

To interpret metal toxicity results at different pH values, it is important to know what changes have occurred in the free metal ion pool before conclusions about the pH dependence of metal toxicity can be made (Peterson et al 1984). The speciation of Cu in the test water at pH 5.7 and 6.5 was predicted using the geochemical speciation model HARPHRQ. The input parameters for HARPHRQ were based on physico-chemical data measured from the test water. Measured concentrations of the inorganic components of Magela Creek water typically deviated <10% from nominal concentrations (appendix F). At pH 5.7, Cu is predicted to occur as the free hydrated metal ion (Cu^{2+} = 98%) with only a very small contribution (1.6%) from $CuOH^{+}$. It is generally accepted that metal toxicity is governed by the activity of the free hydrated metal ion, rather than total Cu concentration (ie the free-ion activity model, FIAM) (Campbell 1995). An exception to the FIAM is lipid-soluble metal complexes; however, such complexes typically represent <1% of the total dissolved metal concentration (Phinney & Bruland 1994). Also, the rate at which a metal complex dissociates at the solution/membrane interface can influence toxicity (Florence & Stauber 1991). Therefore, at pH 5.7 Cu is potentially in its most bioavailable, and hence toxic, form to *Chlorella* sp. Increasing the pH of the test medium to 6.5 reduced the predicted percentage of Cu^{2+} to 89% while the proportion of $CuOH^{+}$ increased to 9.1%. Since ionic Cu is highly correlated with toxicity, then theoretically, Cu should be slightly more toxic to *Chlorella* sp at pH 5.7 than at pH 6.5. However, the results from the growth inhibition tests showed that Cu was more toxic at pH 6.5.

These toxicity results may be better explained with reference to the reported relationship between hydrogen ion concentration (H^+) and metal toxicity. There is growing evidence to suggest that hydrogen ions may decrease the toxicity of metals by competitively excluding them from binding to cell-surface ligands (Peterson et al 1984, Campbell & Stokes 1985, Parent & Campbell 1994). For example, Pawlik et al (1993) found Cd stress to the cyanobacterium *Synechocystis aquatilis* greater at pH 7.0 than at pH 5.5, corresponding to increased uptake of the metal.

In order to investigate the hypothesis that hydrogen ions can affect metal toxicity by directly competing with free metal ions for cellular uptake sites, the cellular metal content of the alga (both intracellular Cu and extracellular Cu) was determined at the completion of the toxicity test at both pH values. Based on this hypothesis, one would expect greater metal uptake at pH 6.5, as a consequence of reduced competition with H^+ at the cell surface.

Uptake of Cu by *Chlorella* sp at pH 5.7 and 6.5 is summarised in table 12. The cellular Cu content (ie intracellular + extracellular Cu) of *Chlorella* sp increased with increasing Cu at both pH 5.7 and 6.5. For example, increasing the Cu concentration from 10 µg Cu L^{-1} to 80 µg Cu L^{-1} resulted in a three-fold increase in intracellular Cu. On a per cell basis, the ratio of extracellular to intracellular Cu was also similar at both pH values. At 10 µg Cu L^{-1}, up to 60% of the total cellular Cu was located intracellularly, and 40% was bound to the cell surface. At the higher Cu concentrations tested, the reverse was typically found.

Table 12 Intracellular and extracellular Cu in *Chlorella* sp in relation to pH

pH 5.7				pH 6.5			
		Intra-Cu	Extra-Cu			Intra-Cu	Extra-Cu
(Cu) µg L^{-1}		(x10 $^{-6}$ng cell^{-1})		(Cu) µg L^{-1}		(x10 $^{-6}$ng cell^{-1})	
10	Mean	5.13	3.54	10	Mean	10.9	8.76
	SD	0.26	0.63		SD	2.05	5.76
80	Mean	18.1	29.0	80	Mean	31.8	48.0
	SD	0.76	11.0		SD	4.57	16.1
640	Mean	121	352	160	Mean	69.4	103
	SD	33.7	314		SD	12.7	9.22

A direct comparison of the cellular Cu content at pH 5.7 and pH 6.5 could be made for the 10 µg Cu L^{-1} and 80 µg Cu L^{-1} treatments. The higher Cu concentrations of 160 µg Cu L^{-1} and 640 µg Cu L^{-1} were derived based on the different EC_{50} values at each pH and therefore could not be directly compared. Extracellular Cu was significantly ($P \leq 0.05$) higher at pH 6.5 than at 5.7, with approximately two times more Cu bound to the cell surface at pH 6.5. This supports the hypothesis that H^+ may competitively exclude Cu from binding at the cell surface, as less Cu was bound to the cell at higher H^+ concentrations. The intracellular Cu content of *Chlorella* sp was also significantly ($P \leq 0.05$) higher at pH 6.5 than at pH 5.7. A two-fold increase in intracellular Cu was measured. These results correspond well with the toxicity results, indicating that increased Cu toxicity to *Chlorella* sp at pH 6.5 was due to increased metal uptake into the cell.

A mass balance of the Cu added to the test containers showed that a considerable amount of Cu was adsorbed to the glass walls of the flasks over 72 h (appendix G). At 10 µg Cu L^{-1}, up to 50% of the Cu added was lost by adsorption. The percentage loss progressively decreased

with increasing Cu concentrations, probably as a result of a saturation of binding sites on the glass walls of the flasks.

Although these flasks were silanised prior to use, it is evident that this aspect of the toxicity test design needs to be improved. An investigation of more suitable test containers may be required.

4.3.4 Discussion

Based on 72 h EC50 values, the toxicity of Cu to Chlorella sp increased markedly with increasing pH from 5.7 to 6.5, supporting previous observations in the literature. Peterson et al (1984) found that total Cu toxicity to the unicellular green alga Scenedesmus quadricauda, increased almost 76-fold with an increase in pH from 5.0 to 6.5. For Chlamydomonas reinhardtii, toxicity of Cu was greater at pH 7 than at pH 5 (Macfie et al 1994). These authors concluded that H+ in solution offered some protection against the toxic metal ions by competing for the same cellular binding sites. The mechanism of this competition is not fully understood. It has been suggested that, as H+ ions increased, they were able to displace Cu from the cell surface, resulting in decreased metal uptake, and hence, decreased toxicity (Meador 1991). However, only a few studies have related pH-dependent toxicity with cellular metal content in algae (Macfie et al 1994, Parent & Campbell 1994). Possible mechanisms of H+ interaction with the metal ion at the algal cell surface are discussed in further detail in Chapter 6.

5 Toxicity of uranium to *Chlorella* sp

5.1 Significance of uranium toxicity testing in tropical Australia

A water quality guideline for U does not currently exist for the protection of freshwater ecosystems in Australia. This is largely the result of limited research in both Australia and abroad. An interim guideline of 5 μg L^{-1} has been set by the Northern Territory Department of Mines and Energy (NTDME 1982) for the protection of freshwater biota in the Magela Creek system, which is located close to Ranger uranium mine. Given the current state of U mining in tropical Australia, there is a fundamental need to assess the potential impacts of U on freshwater biota. Unicellular algae are an important component of the wetland and billabong systems of tropical Australia (Padovan 1992).

5.2 Toxicity of uranium to *Chlorella* sp at pH 5.7

A range-finding test for U was not performed. Instead, concentrations were chosen for the definitive tests based on those used by Markich and Camilleri (1997) for the green hydra *H. viridissima*. The high sensitivity of this species to Cu under similar test conditions allowed a valid comparison to be made with *Chlorella* sp.

Uranium concentrations tested ranged from 8.0 to 265 μg U L^{-1}. Summary data for each U toxicity test are given in appendix D. Acceptable control growth rates of about 1.10 doublings d^{-1} were obtained for each toxicity test, with coefficients of variation of <20%. Continuous exponential growth was maintained in all control treatments over 72 h and the toxicity test was very reproducible between individual test-runs over 72 h.

Growth of *Chlorella* sp typically decreased with increasing U concentration. The lowest concentration of U that significantly ($P \leq 0.05$) reduced algal growth was 64 μg U L^{-1}. Uranium concentrations up to 34 μg L^{-1} had no significant ($P > 0.05$) effect. The 72 h EC_{50} values for each toxicity test ranged from 70 to 79 μg U L^{-1} (table 13). Since the 95% confidence intervals from each test overlapped, pooled data were used for the derivation of BEC_{10}, MDEC and mean EC_{50} values. Measured U concentrations were typically 15–20%

lower than nominal concentrations, indicating a considerable loss of U to the glass walls of the test flasks, despite silanisation prior to the test. pH control in the unbuffered water was reasonable over 48 h, typically rising <0.5 pH units. However, as a result of high cell densities in the controls and several of the lower U treatments, pH increased to about 6.5 by day 2 and 3 of the toxicity test.

A combined concentration-response curve of the toxicity of U to *Chlorella* sp (at pH 5.7), together with calculated endpoints, is shown in figure 12. The concentration-response curve was particularly steep from about 60 µg U L^{-1}, with relatively small changes in U concentrations causing pronounced growth inhibition. Unlike the results obtained in the presence of Cu, *Chlorella* sp showed a typical sigmoidal response in the presence of increasing U concentrations. The 72 h EC_{50} value was 78 µg U L^{-1}, with 95% confidence intervals of 71–83 µg U L^{-1}. This value is consistent with the mean 72 h EC_{50} value of 75 µg U L^{-1} derived from the individual test-runs. The BEC_{10} and MDEC values were 21 µg U L^{-1} and 34 µg U L^{-1} respectively, which are lower than the NOEC and LOEC values estimated by the hypothesis testing approach (table 13).

Table 13 Summary of test endpoints on U toxicity to *Chlorella* sp at pH 5.7

Test-run	Mean cell division rate (doublings d^{-1})	U toxicity (µg L^{-1})		
		72 h EC_{50} (95% CI)	NOEC	LOEC
1st definitive	1.00	70 (65–75)	33	65
2nd definitive	1.11	77 (70–85)	34	67
3rd definitive	1.12	79 (73–86)	16	64

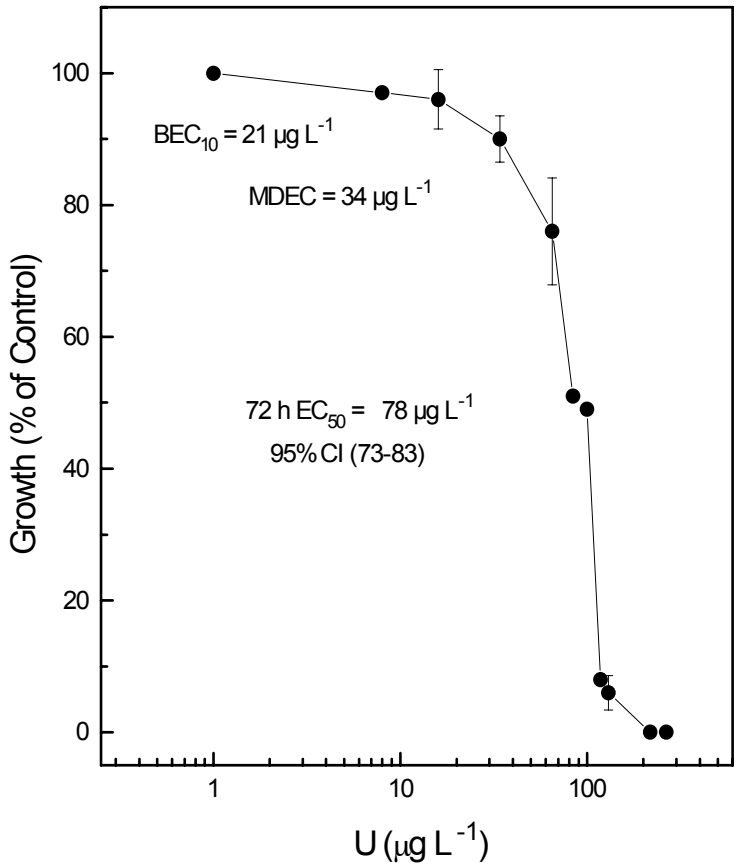


Figure 12 Growth inhibition of *Chlorella* sp exposed to U over 72 h at pH 5.7. Data points represent the mean of three tests ± one SD (except for 8.0, 33, 217 and 265 µg Cu L^{-1}).

Since a typical sigmoidal response was obtained for *Chlorella* sp in the presence of U, the EC_{50} was considered to be an appropriate test endpoint. The data were also re-analysed over 48 h to assess the possibility of shortening the toxicity test without compromising the sensitivity and statistical significance of the test. Although good control growth rates were obtained over 48 h, test results were more variable with significantly ($P \leq 0.05$) different 48 h EC_{50} values (ie 95% CI not overlapping) between individual test-runs (appendix E).

5.3 The influence of pH on uranium toxicity

It has already been established for *Chlorella* sp that the toxicity of Cu is highly pH-dependent. Results obtained revealed marked increases in Cu toxicity at pH 6.5 compared to 5.7, due to increased Cu uptake into the cells. A review of the literature on U toxicity reveals a similar pH-dependence, although, very few studies have been conducted. Hence, one objective of the present study was to assess the influence of pH changes on the toxicity of U to the freshwater alga *Chlorella* sp.

5.3.1 Toxicity of U to *Chlorella* sp at pH 6.5

Uranium concentrations used for toxicity testing at pH 6.5 were based on those used for testing at pH 5.7, covering a concentration range from 8.0–265 µg U L^{-1}. Because of the increased sensitivity to Cu detected at pH 6.5, an additional test concentration of 4 µg U L^{-1} was used. Summary data for each toxicity test are given in appendix D. Good cell division rates of about 1.60 doublings d^{-1} were obtained in all controls, with coefficients of variation of <20%, indicating test acceptability.

Chlorella sp showed increased sensitivity to U at pH 6.5. Three separate 72 h toxicity tests gave consistent EC_{50} values from 45 to 48 µg U L^{-1} (table 14). Since the 95% confidence intervals from each test overlapped, these values were not considered significantly different. NOEC and LOEC values of 8.0 and 17 µg U L^{-1} respectively were calculated. Measured U concentrations at the beginning of the toxicity tests were considerably lower (15–20% deviation) than nominal concentrations. pH control in the unbuffered water was reasonable throughout the toxicity tests, rising by <0.5 pH units over 72 h. However, control treatments and some of the lower U treatments exceeded this range by day 2 of the test, rising to as high as pH 7.2.

Table 14 Summary of test endpoints on U toxicity to *Chlorella* sp at pH 6.5

Test-run	Mean cell division rate (doublings d^{-1})	U Toxicity (µg L^{-1})		
		72 h EC_{50} (95% CI)	NOEC	LOEC
1st definitive	1.62	48 (39–60)	<18	18
2nd definitive	1.56	45 (40–50)	9.0	18
3rd definitive	1.62	46 (41–51)	8.0	17

Combined data from the three toxicity tests was used to plot a concentration-response curve. A typical sigmoidal relationship was found for *Chlorella* sp at pH 6.5 with increasing U concentrations (fig 13). At concentrations >8 µg U L^{-1}, small increases in U caused pronounced growth inhibition. The 72 h EC_{50} value was 44 µg U L^{-1} with 95% confidence intervals of 39–49 µg U L^{-1}. The BEC_{10} and MDEC values were 11 µg U L^{-1} and 13 µg U L^{-1} respectively. A comparison of the test results over 48 h detected a significant difference among EC_{50} values between individual test-runs (ie 95% CI not overlapping) (appendix E).

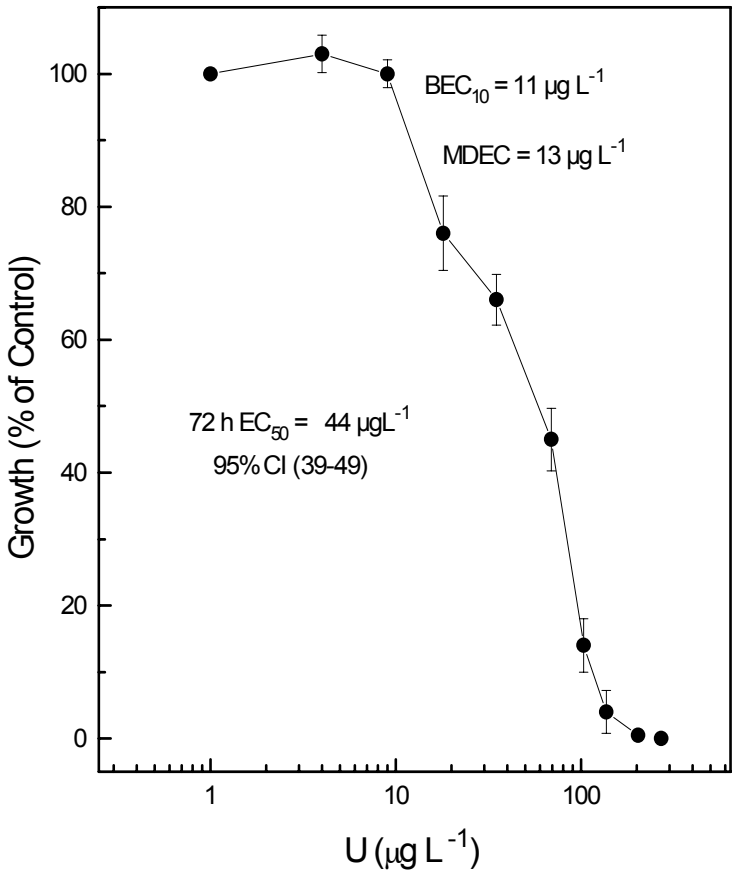


Figure 13 Growth inhibition of *Chlorella* sp exposed to U over 72 h at pH 6.5. Data points represent the mean of three tests ± one SD (except for 4.0, 205, and 271 µg Cu L^{-1}).

5.3.2 Comparison of U toxicity to *Chlorella* sp at pH 5.7 and pH 6.5

Uranium was significantly ($P \leq 0.05$) less toxic to *Chlorella* sp at pH 5.7 than at pH 6.5. Increasing the pH of the test medium resulted in a reduction in the 72 h EC_{50} value from 78 µg U L^{-1} to 44 µg U L^{-1} (table 15). Based on the BEC_{10} and MDEC results, a two-fold reduction in U toxicity was also observed as the pH decreased from 6.5 to 5.7. To interpret these results requires an understanding of how the chemical speciation of U varies with pH.

Table 15 Comparison of test endpoints for *Chlorella* sp at pH 5.7 and pH 6.5

	U toxicity (µg L^{-1})	
	pH 5.7	**pH 6.5**
72 h EC_{50} (95% CI)	78 (71–83)	44 (39–49)
BEC_{10}	21	11
MDEC	34	13

The speciation of U in the test water at pH 5.7 and 6.5 was predicted using HARPHRQ (see section 4.3.3). Unlike the relatively simple speciation distribution predicted for Cu at pH 5.7 and 6.5, the speciation of U in the test water is complex. At pH 5.7, the free uranyl ion (UO_2^{2+}) constitutes a minor proportion of the total U concentration (ie 12% at 0.1 µg L^{-1} declining to 1.3% at 1000 µg L^{-1}). In contrast, polymeric uranyl species, including $(UO_2)_3(OH)_5^+$, $(UO_2)_3(OH)_7^-$ and $(UO_2)_2(OH)_3CO_3^-$ increase in significance with total U concentration. This increase is compensated by a reduction in the relative proportions of

monomeric uranyl species (ie $UO_2(OH)^+$ and UO_2CO_3). Increasing the pH of the test medium to 6.5 increased the formation of polymeric uranyl species. The proportion of free uranyl ion (UO_2^{2+}) was found to be insignificant at pH 6.5 (<1%). Markich et al (1996) provided evidence that the toxicity of U to biota is governed by UO_2^{2+} and UO_2OH^+, rather than the sum of inorganic uranyl species. These uranyl species are considered toxic because they are thought to be more biologically active than uranyl carbonate complexes which predominate at higher pH levels (Poston et al 1984).

It is apparent from the 72 h EC_{50} values obtained in the present study, that the growth response of *Chlorella* sp is not solely dependent on the speciation of U in the test waters over the pH range 5.7–6.5. If this were true, then there should be greater toxicity at pH 5.7 compared to pH 6.5, corresponding with a greater proportion of UO_2^{2+} and UO_2OH^+. However, the results from the growth inhibition tests showed that U was more toxic at pH 6.5. Like Cu, these U toxicity results may be better explained in terms of competition between H^+ and the metal at the cell surface. This hypothesis was investigated by determining the cellular U content of *Chlorella* sp at the completion of the toxicity test at both pH values.

Uptake of U by *Chlorella* sp at pH 5.7 and 6.5 is summarised in table 16. The cellular U content (ie intracellular + extracellular U) increased with increasing U concentration at both pH 5.7 and 6.5. For example, increasing the U concentration from 40 µg L^{-1} to 250 µg L^{-1} resulted in an eight-fold increase in both intracellular and extracellular U (pH 5.7 and pH 6.5). On a per cell basis, the ratio of extracellular to intracellular U was similar at both pH values, with less than 30% of the total cellular U located intracellularly.

Table 16 Intracellular and extracellular U in *Chlorella* sp in relation to pH

pH 5.7				pH 6.5			
		Intra-U	Extra-U			Intra-U	Extra-U
(U) µg L^{-1}		(x10 $^{-6}$ng cell^{-1})		(U) µg L^{-1}		(x10 $^{-6}$ng cell^{-1})	
40	Mean	16.8	21.7	40	Mean	31.1	70.5
	SD	3.6	10.7		SD	3.8	26.1
120	Mean	69.6	116	120	Mean	90.9	196
	SD	5.2	39.0		SD	3.0	39.7
250	Mean	135	170	250	Mean	247	543
	SD	6.4	36.8		SD	20.6	121

At all U concentrations tested, intracellular U was significantly ($P \leq 0.05$) greater at pH 6.5 than at pH 5.7, with approximately two times more U located inside the cell. The concentration of extracellular U was also greater at pH 6.5 compared to pH 5.7, however this difference was only significant for the 250 µg U L^{-1} treatment as a result of large variation between replicates.

The Coulter Counter allowed algal cell size distribution to be determined for the 40 and 120 µg U L^{-1} treatments (appendix G). Determination of the mean cell diameter was not possible however for the 250 µg U L^{-1} treatment as most of the cells had begun to die and therefore did not display the typical bell-shaped distribution. Cells exposed to U had a significantly ($P \leq 0.05$) larger mean cell diameter compared to control cells, and this increase in cell surface area was similar at both pH values. Rosko and Rachlin (1977) reported similar swelling of *Chlorella vulgaris* cells in the presence of Cu, which was probably due to an uncoupling of cell growth and cell division (Foster 1977, Florence & Stauber 1986). For

Chlorella sp, this increase in cell size suggests that U may specifically inhibit cell division in this alga. Because U-treated cells were larger than control cells, intracellular and extracellular U was also expressed on a cell surface area basis (appendix G). At 120 µg U L^{-1}, significantly ($P \leq 0.05$) more intracellular and extracellular U was detected at pH 6.5 compared to pH 5.7. This difference was not detected for the 40 µg U L^{-1} treatment on a cell surface area basis due to the high variability in the analyses at this U concentration.

A mass balance of the U added was also calculated. At the higher U concentrations (120 and 250 µg U L^{-1}) most U at the end of the test was still present in solution (>50% dissolved U). The intracellular and extracellular U fractions each represented less than 10% of the total U added. A significant fraction of U adsorbed to the walls of the flasks throughout the test (>20%) despite silanisation of the glass flasks prior to the toxicity test. Alternative test containers for metal toxicity tests should be investigated.

In agreement with the cellular Cu results, the U results support the hypothesis that H^+ may competitively exclude metals from binding at the cell surface. Under this rationale, increased concentration of extracellular U at pH 6.5 would be related to reduced competition between the metal ion and the H^+ at the cell surface. Since more U was bound extracellularly at pH 6.5, a greater concentration of U could therefore be transported across the cell membrane and into the cell to elicit a toxic effect. Unfortunately, no data were available from the literature relating to U toxicity to validate this hypothesis. However, a growing body of evidence has been generated for Cu (see section 4.6.4). Given the similar binding strengths of Cu and U to functional groups on the cell surface (Smith et al 1997), it may be reasonable to assume that a similar mechanism of H^+ competition is taking place.

6 General discussion

Toxicity testing with unicellular algae is increasingly being used in Australia to assess the impact of potential pollutants on aquatic ecosystems. This is largely due to their high sensitivity and reproducibility under laboratory conditions, along with their high ecological significance as the foundation of most aquatic food chains (Stauber 1995). Standard freshwater test species commonly used for routine toxicity testing in Australia include *Selenastrum capricornutum* (now called *Raphidocellus subcapitata*) and *Chlorella protothecoides*, for which standard test protocols have been developed (Stauber et al 1994, USEPA 1994). *S. capricornutum* is not widely distributed in Australia and both *C. protothecoides* and *S. capricornutum* are temperate species. Given the differences in environmental conditions between tropical and temperate regions, and the way in which such conditions may modify toxicity, the ecological relevance of using either of these species for assessing the impact of metals in tropical Australian freshwaters is uncertain. Increased temperature, for example, has been shown to increase the toxicity of several metals to algae (Garnham et al 1992, Pawlik & Skowronski 1994). While a standard toxicity test has been developed using tropical algae (*Chlorella* sp 12) from Papua New Guinea (Stauber unpublished data), no such test is currently available for tropical Australian algae. This causes obvious problems for assessing the toxicity of mine wastewaters in the World Heritage listed Kakadu National Park because the introduction of exotic species into the park is prohibited. Consequently, data with local species are needed to provide a sound basis for aquatic environment protection in this region of Australia.

In this study, a standard tropical *Chlorella* toxicity test, using a new isolate from Kakadu National Park, Northern Territory, was successfully developed with optimal incubation conditions, inoculum age and test media. The standardised test conditions allowed good algal

growth rates of 1.60 ± 0.3 doublings d^{-1} (pH 6.5) to be obtained for *Chlorella* sp, which are comparable to other freshwater algal protocols. The use of a synthetic standard water, supplemented only with minimal nutrients, in the test protocol is consistent with current recommendations for toxicity testing with algae (Stauber et al 1994). In particular, the newly derived protocol avoids the use of complexing agents and high nutrient concentrations, which have long been incorporated into most overseas test protocols (Environment Canada 1992, USEPA 1994). Such media can complex and adsorb metals so that their toxic effect is severely underestimated (Stauber & Florence 1989). The standard test water used for *Chlorella* sp is typical of sandy-braided streams throughout the Australian wet/dry tropics (ie soft, slightly acidic, with a low buffering and complexing capacity). Consequently, these test conditions maximise the toxicity of metals to the test species.

A key aim of this project was to determine the suitability and sensitivity of a new isolate of freshwater algae *Chlorella* sp, for the purpose of monitoring the impacts of mine wastewaters in tropical Australia. A toxicity test with this primary producer was to be added to the suite of freshwater tests currently available using tropical freshwater invertebrates and fish.

The choice of any toxicity test species depends upon its availability, culture requirements and ease of use (Lewis 1995). Based on these criteria, *Chlorella* sp (Kakadu isolate) can be recommended as a standard test species for use in tropical waters. Although the precise distribution of this species is not known, *Chlorella* has been routinely isolated from tropical waters in Australia and Papua New Guinea (PNG), and was shown to be an important part of the flora in the Fly River, PNG (Stauber unpublished data). Unlike species of cyanobacteria and diatoms that are not frequently used in toxicity tests due to their slow growth and demanding culture requirements (Lewis 1995), *Chlorella* sp (Kakadu isolate) grows well under defined laboratory conditions and is relatively easy to culture and maintain. Furthermore, this species can be more easily counted compared to other filamentous and clumping algae. In addition to this alga's considerable value as a laboratory test organism, *Chlorella* sp was highly sensitive to Cu and U. Inhibitory effects on algal growth were observed at Cu concentrations as low as 0.7 µg Cu L^{-1} and U concentrations as low as 13 µg U L^{-1} in low nutrient softwater. Thus, toxicity tests with this species are sensitive enough to detect adverse effects at Cu concentrations close to ANZECC guideline values of 1–5 µg Cu L^{-1}.

The most reliable and sensitive endpoint for the algal toxicity test was 50% growth inhibition (EC_{50}) obtained after 72 h at pH 6.5. An evaluation of alternative endpoints to the EC_{50} was undertaken because of the relatively flat concentration response-curve for Cu. It was concluded that the EC_{25} and EC_{75} were less reliable than the EC_{50} due to significant differences between individual test-runs. One obvious limitation of the toxicity test is that it takes 72 h and is quite labour intensive, because cell densities have to be determined daily. To overcome this, attempts were made to reduce the test to 48 h. Although acceptable control growth rates were obtained (>1 doublings d^{-1}), the data was more variable over 48 h, and therefore, a 48 h test was not considered a viable option. It was also concluded that the algal toxicity test should be carried out at pH 6.5 rather than pH 5.7 due to the increased sensitivity of the alga to both Cu and U. This will provide the 'greatest risk' scenario in terms of assessing the impact of Cu and U to this alga.

It was difficult to compare the toxicity of Cu to *Chlorella* sp with various freshwater algal species because most toxicity tests were carried out in culture media containing chemicals which complex Cu, thus underestimate its toxicity. However, several comparable studies were available. Stauber and Florence (1989) reported a 72 h EC_{50} value of 16 µg Cu L^{-1} for the temperate freshwater alga *Chlorella protothecoides* in synthetic softwater (pH 7.5). In high nutrient MBL medium, the same authors reported a 72 h EC_{50} value of >200 µg Cu L^{-1},

emphasising the importance of the test medium in deriving toxicity data. For the tropical freshwater isolate Chlorella sp 12 from Papua New Guinea, a 72 h EC_{50} value of 8.4 µg Cu L^{-1} was obtained in synthetic softwater (see section 4.2). It should be noted that all these studies were performed at pH 7.5, and therefore, are not directly comparable to this study with *Chlorella* sp because Cu toxicity is highly pH dependent. However, given the constraints in making such a comparison, the study shows that *Chlorella* sp is as sensitive as other freshwater algae to Cu.

The relative sensitivity of *Chlorella* sp to Cu was compared with other tropical freshwater biota. For comparative purposes, only toxicity data that were derived under similar environmental conditions (ie softwater, slightly acidic, low alkalinity and conductivity) are used (table 17). For the green hydra (*Hydra viridissima*), Markich and Camilleri (1997) reported a 96 h EC_{50} value for growth inhibition of 4.0 µg Cu L^{-1} in synthetic Magela Creek water (pH 6.0). The same authors reported a 96 h LC_{50} value of 23 µg Cu L^{-1} for the toxicity of Cu to the sac-fry of the gudgeon fish *Mogurnda mogurnda*. Although this 'lethality' endpoint is not directly comparable to the more sensitive 'sublethal' EC_{50} endpoint, the authors applied an extrapolation factor based on work by Hendriks (1995), giving an EC_{50} value of 11 µg Cu L^{-1}. Giles (1974) provided toxicity data for the freshwater prawn *Macrobrachium* sp in natural Magela Creek water (pH 7.0), reporting an 96 h LC_{50} value of 170 µg Cu L^{-1}. This value, however, may not be comparable because the author did not measure the organic carbon content in the water, which is known to complex Cu. Consequently, much of the Cu may have been complexed to organic material in the water thereby reducing its bioavailability, and hence toxicity, to the test species. Recent investigations on the valve movement behaviour of the tropical freshwater bivalve *Velesunio angasi* reported a 48 h EC_{50} value of 10 µg Cu L^{-1} (pH 6.0) in synthetic Magela Creek water (Markich unpublished data).

Table 17 Comparative toxicity of Cu to Australian tropical freshwater biota in synthetic Magela Creek water

Species	Endpoint	Cu toxicity		Reference
		µg L^{-1}	µ mol L^{-1}	
Unicellular algae (*Chlorella* sp)	72 h EC_{50}	35 (pH 5.7) 1.5 (pH 6.5)	0.55 0.02	This study
Cnidaria (*H. viridissima*)	96 h EC_{50}	4.0 (pH 6.0)	0.06	Markich & Camilleri (1997)
Chordata (*M. mogurnda*)	96 h LC_{50}	23 (pH 6.0)	0.36	Markich & Camilleri (1997)
Mollusca (*V. angasi*)	48 h EC_{50}	10 (pH 6.0)	0.16	Markich (unpublished data)

The relative sensitivity of these various test species to Cu is best compared at pH 5.7–6.0, over which range the speciation of Cu does not differ significantly ($P > 0.05$). Based on EC_{50} values, *Chlorella* sp was less sensitive to Cu compared with the green hydra, the bivalve and the gudgeon fish. However, *Chlorella* sp was inhibited at concentrations as low or lower than the other tropical species mentioned (ie MDEC values (µg Cu L^{-1}): 1.5 (alga), 1.6 (hydra), 6.7 (bivalve), 7.0 (fish)). Furthermore, *Chlorella* sp was extremely sensitive to Cu at pH 6.5 (72 h EC_{50} = 1.5 µg Cu L^{-1}). Further work is needed to address the influence of pH on the toxicity of Cu to other tropical freshwater species.

This study provides the first data on the toxicity of U to a freshwater alga. The 72 h EC_{50} value was 78 µg U L^{-1} at pH 5.7 and 44 µg U L^{-1} at pH 6.5. Although there are no comparable toxicity data on the effects of U on unicellular algae, there is some data for Australian tropical animals (table 18). Using the same water quality conditions as this study, Markich et al (1996)

reported a 48 h EC_{50} value of 254 µg U L^{-1} for the freshwater bivalve *V. angasi* based on valve behaviour of the organism (at pH 5.8). Uranium was also found to be less toxic to the green hydra (*H. viridissima*) and gudgeon fish (*M. mogurnda*), with EC_{50} values of 95 and 1360 µg U L^{-1} (96 h LC50 value ÷2 for comparison), respectively (pH 6.0) (Markich & Camilleri 1997). Comparable data were available at pH 6.5 for the water flea *Moinodaphnia macleayi*, with a 120 h EC_{50} value of 39 µg U L^{-1} (3 brood reproduction test) (Markich & Camilleri 1997). Despite the different test durations used, the order of sensitivity to U appears to be:

Unicellular algae ≥ Crustacea > Cnidaria > Mollusca > Chordata.

Table 18 Comparative toxicity of U to Australian tropical freshwater biota in synthetic Magela Creek water

Species	Endpoint	U toxicity			Reference
		µg L^{-1}		µ mol L^{-1}	
Unicellular algae (*Chlorella* sp)	72 h EC_{50}	78 44	(pH 5.7) (pH 6.5)	0.33 0.18	This study
Crustacea (*M. macleayi*)	120 h EC_{50}	39	(pH 6.5)	0.16	Markich & Camilleri (1997)
Cnidaria (*H. viridissima*)	96 h EC_{50}	95	(pH 6.0)	0.40	Markich & Camilleri (1997)
Chordata (*M. mogurnda*)	96 h LC_{50}	1360	(pH 6.0)	4.8	Markich & Camilleri (1997)
Mollusca (*V. angasi*)	48 h EC_{50}	254	(pH 5.8)	1.1	Markich (unpublished data)

It should be noted that this trend is based on only one species from each phylum and thus may not be a true indication of the relative toxicities when multiple species are compared. However, it does suggest that *Chlorella* sp is a very sensitive test organism among the tropical freshwater biota, and therefore, would be a good indicator of metal contamination in mine wastewaters entering Magela Creek, downstream of Ranger uranium mine.

Based on EC_{50} values (expressed as µg L^{-1}), *Chlorella* sp is approximately two times more sensitive to Cu than U at pH 5.7, and up to 30 times more sensitive to Cu at pH 6.5. However, if the toxicity of these two metals is compared on a molar basis (ie to account for the difference in molecular mass), U is nearly two times more toxic than Cu at pH 5.7. At pH 6.5, Cu is the more toxic (>8) of the two metals. The toxicity results for Cu and U at pH 5.7 are anomalous when compared to toxicity data for temperate and tropical biota typically where the toxicity of U was relatively low compared to Cu (Poston et al 1984, Markich & Camilleri 1997). No explanation can be given for the comparatively high sensitivity of *Chlorella* sp to U. However, it is evident from the concentration-response curve obtained for U and Cu that this alga responds differently to the two metals. In the presence of U, *Chlorella* sp showed a sigmoidal-type response, resulting in a sharp decline in algal growth above a certain threshold concentration. This response is typical of most organisms in the presence of a toxicant. However for Cu, the concentration-response curve was particularly flat over a wide concentration range (20–160 µg Cu L^{-1}, fig 7), indicating that increasing the Cu concentration had little additional effect on algal growth. It is possible that this flat concentration-response curve may be due to detoxification of Cu by the algal cells. Certain algal species, including *Scenedesmus actutiformis* and *Chlorella fusca*, have been shown to produce organic substances that reduce biologically available Cu if they are released extracellularly in sufficient amounts (Robinson 1989). Cell wall exclusion of Cu and intracellular detoxification by binding to phytochelatins or polyphosphate has also been demonstrated in a variety of

freshwater algal species in response to elevated Cu concentrations (Twiss et al 1993, Knauer et al 1997).

Further experimental work is required to explain the unusual concentration-response curve for this alga in the presence of Cu. This could be achieved by determining the intracellular metal content of the alga at various concentrations within the flat part of the concentration-response curve (ie 10, 20, 40, and 80 µg Cu L^{-1}). If Cu is being detoxified outside the cell or excluded from the cell due to extracellular binding with exudates or membrane sites, then the intracellular metal content would be the same over this range of Cu concentrations. Alternatively, if the alga is capable of immobilising Cu intracellularly by binding to phytochelatins, thiols etc, then intracellular Cu concentrations would increase over this Cu concentration range.

The toxicity of Cu and U is markedly affected by key water quality variables, such as pH, water hardness and organic carbon concentration. In natural waters, these factors modify toxicity by either increasing or decreasing the concentration required to produce a particular biological response (Chapman 1995). This study focused on the effect of pH on the toxicity of Cu and U to *Chlorella* sp. Based on EC_{50} values, an 18-fold increase in Cu toxicity and a two-fold increase in U toxicity was observed by increasing the pH from 5.7 to 6.5. A two-fold increase in metal toxicity was observed on the basis of BEC_{10} and MDEC values over the same pH range. Both of these pH values are within the range that occur in tropical waterbodies receiving metals at elevated concentrations from anthropogenic sources. Determination of cellular metal content of the alga demonstrated that significantly more Cu and U was bound at the algal cell surface and transported intracellularly at pH 6.5 compared to pH 5.7, corresponding to greater growth inhibition. These results support a growing body of evidence that H^+ in solution offers some protection against metal ions (Crist et al 1988, Schenck et al 1988, Parent & Campbell 1994). The exact mechanism is as yet unexplained, but supporting evidence for a number of hypotheses is available.

The effect of pH on metal toxicity is two-fold. The H^+ concentration may exert its effect either directly by affecting metal uptake or indirectly by affecting the chemical speciation of the dissolved metal pool (Peterson et al 1984). Much of the confusion in the literature surrounding metal-pH toxicity effects stems from an inability to distinguish between these two factors. Numerous studies have interpreted their results solely in terms of changes in metal speciation in solution, ignoring the possibility of metal-H^+ interaction (Helliwell et al 1983, Starodub et al 1987). Determination of Cu speciation in this study confirmed that speciation changes were only minimal between pH 5.7 and 6.5 (ie <10% decrease in Cu^{2+} from pH 5.7 to 6.5). Also, the use of a simple inorganic medium minimised the effects of variables other than pH, such as metal complexation, so that changes in toxicity reflected, to a large extent, changes related only to H^+ concentration.

Several possible mechanisms to explain the apparent protective effect of the proton H^+ at decreasing pH are available. These include competition between H^+ and the free metal ion at the cell surface and changes in algal surface potential or membrane permeability.

The surfaces of algae contain a number of functional groups with high affinity for metal ions and carry a net negative charge, mainly due to carboxylic, sulfhydryl, and phosphatic groups (Rai et al 1981, Crist et al 1990; 1994). These groups comprise binding sites that transport metal ions across the cell membrane and into the cell. It is suggested that H^+ can compete with the ionic form of the metal at the cell surface for available binding sites. Parent and Campbell (1994) provided direct evidence for H^+/Al^{3+} competition for the green alga, *Chlorella pyrenoidosa* (renamed *C. protothecoides*). In a series of acid titrations, algal cells were shown

experimentally to take up more H^+ at pH 4.3 than at 6.5, corresponding to reduced concentrations of cell-bound and intracellular Al. These authors, however, concluded that competition at the cell surface could only explain part of the pH-dependent toxicity.

Changes in algal surface potential have also been implicated as a possible mechanism for increased metal toxicity at increasing pH. Crist et al (1988) demonstrated that as the pH of the medium is increased, an increasing number of negatively charged sites are formed on the algal surface. Because the interaction between metals and algal surfaces involves electrostatic bonding, this may result in increased metal adsorption to the cell surface, hence, greater uptake and toxicity. Alternatively, conformational changes in metal binding sites at the cell surface may occur, thereby altering the cell binding capacity for the metal (Parent & Campbell 1994).

It has also been suggested that H^+ may interact with the plasma membrane (Macfie et al 1994) thereby altering its permeability to certain metals, although there is no direct evidence to support this hypothesis. Such a mechanism assumes that transport of the metal across the plasma membrane is a prerequisite for toxicity. While most studies have concluded that the mechanism of Cu toxicity to algae involves some form of structural alteration (Wong et al 1994), or disturbance of the ratio of oxidised to reduced glutathione within the cell, leading to reduced cell division (Stauber & Florence 1987), it has also been suggested that interference with the cell membrane is a fundamental step in the sequence of cupric ion toxicity to algae (Gross et al 1970, Sunda & Huntsman 1983). Alternatively, if we accept that Cu enters the cell by facilitated diffusion and exerts its toxic effect either in the cytosol or in the cell membrane, the pH effect can be explained simply by the extent and rate of formation of the Cu-carrier protein(s)(P) complex(s) (Florence & Stauber 1991).

The increased toxicity of Cu and U to *Chlorella* sp at pH 6.5 is considered to be a function of one, or possibly the interaction of all of the above mentioned mechanisms. However, in more complex situations, such as natural water, this relatively simple relationship between H^+ and metals is likely to be masked by other related factors that also vary with pH, the most prominent of which are metal speciation and organic metal complexation. Organic complexation of metals generally increases with pH, and metal solubility decreases causing a reduction in the free ion pool (Peterson et al 1984, Apte & Day 1993). Because of these related factors, the effects of acidification at the biological surface are sometimes found to be less important than are its effects on metal speciation in solution. Consequently, the ability to satisfactorily predict the way in which pH may modify the toxicity of metals to biota in natural waters may be limited.

7 Improvement of initial test protocol for *Chlorella* sp and determination of the reliability of *Chlorella* sp as a standard test organism

7.1 Incorporation of HEPES buffer into the initial test protocol for *Chlorella* sp

The newly developed test protocol for *Chlorella* sp was considered appropriate for use in routine toxicity testing as it is ecologically relevant and sensitive. In particular, the use of a synthetic softwater as the test medium maximises the toxicity of metals to the test species. One inherent limitation of the toxicity test however, was the difficulty in maintaining the pH over the entire test in the poorly buffered test water. Initially, the use of a pH buffer was

avoided because of reports that buffers interfere with the growth of the test species and also with metal toxicity (Lage et al 1996, L Hales pers comm). An alternative method of adjusting the pH twice a day was instead incorporated into the initial test protocol, and this proved to be effective in maintaining the pH over the first 48 h of the test. The effectiveness of this method was largely diminished over the last 24 h of the test, thereby compromising the validity of the results. Furthermore, this method was quite labour intensive and tedious. This prompted an investigation to identify the suitability of using HEPES buffer at 2 mM and 5 mM for metal toxicity testing with *Chlorella* sp. HEPES buffer was chosen because it has been used previously in algal toxicity tests. Other buffers may be more suitable in the pH range tested (ie MES pKa = 6.1) however this was not investigated.

The growth of control cultures of *Chlorella* sp in the presence of 2 mM and 5 mM HEPES, but in the absence of Cu and U, was initially evaluated to confirm the lack of possible toxic effects on the algae. At 2 mM and 5 mM concentrations, HEPES did not significantly ($P > 0.05$) interfere with the growth of *Chlorella* sp compared to unbuffered synthetic softwater (table 19).

Table 19 The influence of HEPES buffer on cell division rates of *Chlorella* sp exposed to Cu at pH 6.5

HEPES buffer (mM)	Mean control cell division rate (doublings d^{-1})	72 h EC_{50} ($\mu g\ Cu\ L^{-1}$)		pH*	
				(day 0)	(day 3)
0 (Control)	1.62	3.6	(2.8–4.6)	6.5	7.1
2	1.48	4.0	(2.5–6.2)	6.5	6.8
5	1.54	13.7	(10.8–17.3)	6.5	6.6

* typical pH range without correction over 3 d

The influence of Cu on *Chlorella* sp growth rates in 2 mM and 5 mM HEPES-buffered media is shown in appendix H. For comparison, the results obtained in the unbuffered medium were also included in the same figure. In all media, growth of the alga typically decreased with increasing Cu concentration. The 72 h EC_{50} value in the unbuffered synthetic softwater was 3.6 $\mu g\ Cu\ L^{-1}$ (CI 2.8–4.6 $\mu g\ L^{-1}$). The addition of 2 mM HEPES to the test medium did not significantly ($P > 0.05$) interfere with Cu toxicity to *Chlorella* sp compared to the unbuffered medium, with a similar 72 h EC_{50} value of 4.0 $\mu g\ Cu\ L^{-1}$ (CI 2.5–6.2 $\mu g\ L^{-1}$). Copper toxicity was significantly ($P \leq 0.05$) lower in the presence of 5 mM HEPES than in the unbuffered medium, as indicated by an increase in the 72 h EC_{50} from 3.6 to 13.7 $\mu g\ L^{-1}$ (CI 10.8–17.3 $\mu g\ L^{-1}$). This decrease in toxicity in the presence of 5 mM HEPES was possibly due to complexation of the buffer with the metal in solution, thereby decreasing its bioavailability to the test species. At 2 mM HEPES, metal complexation was considered negligible due to the similar toxic response obtained compared to the unbuffered control.

Typical pH ranges over the 72 h toxicity test, with and without the addition of HEPES, are shown in table 19. In the unbuffered control medium, pH control was fairly poor, rising >0.5 pH unit from pH 6.5 on day 3 of the test. The 2 mM and 5 mM HEPES-buffered media were effective in maintaining the pH close to pH 6.5 over 72 h, rising less than 0.5 pH unit throughout the test. Tighter pH control was obtained with the addition of 5 mM HEPES (0.1 pH unit increase) compared to 2 mM HEPES (0.3 pH unit increase).

The influence of HEPES buffer on U toxicity was also investigated. Since the 5 mM HEPES-buffered media was shown to alter Cu toxicity, it was considered inappropriate to use in the toxicity test and therefore only the 2 mM HEPES-buffered media was tested for U. At this concentration, HEPES did not significantly ($P > 0.05$) interfere with the growth of *Chlorella* sp which had a cell division rate of 1.53 doublings d^{-1} similar to the unbuffered

media (1.49 doublings d^{-1}) (table 20). Similar concentration-response curves for U were obtained with and without the addition of HEPES (appendix H). The 72 h EC_{50} value in the 2 mM HEPES-buffered medium was 10.5 µg L^{-1}, which was not significantly ($P > 0.05$) different to the unbuffered medium (11.3 µg L^{-1}). Good pH control was obtained with the addition of 2 mM HEPES, rising <0.5 pH units over the 72 h toxicity test.

Table 20 The influence of HEPES buffer on cell division rates of *Chlorella* sp exposed to U at pH 6.5

HEPES buffer (mM)	Mean control cell division rate (doublings d^{-1})	72 h EC_{50} (µg U L^{-1})	pH*	
			(day 0)	(day 3)
0 (Control)	1.49	11.3 (8.2–15.6)	6.5	7.1
2	1.53	10.5 (8.2–13.3)	6.5	6.8

* typical pH range without correction over 3 d

7.1.1 Discussion

For pH buffers to be considered for use in metal toxicity testing, it is essential that they fulfil the following requirements: a) the absence of toxicity to the test species, b) the absence of complexation with the metals of interest, and c) be effective in their role of maintaining the pH of the medium close to the required level. A review of the literature relating to the suitability of buffers in metal toxicity studies has found that under many circumstances, at least one of these properties is not met. Lage et al (1996) showed that addition of HEPES buffer (25 mM at pH 8.0) to the test medium significantly increased Cu toxicity (synergistic effect) to the marine dinoflagellate *Amphidinium carterae*. The buffer TRIS (tris(hydroxymethyl)amino methane) has been shown to interfere with the growth of several phytoplankton (Harrison et al 1980), in addition to complexing Cu (Gavis et al 1981). Other pH buffers used in toxicity studies have been shown to complex Cu, including TEA (2,2'2"-nitrilotriethanol) and the alkaline buffer DIPSO (3-[N,N-bis(2-Hydroxyethyl)amino]-2-hydroxypropane-sulfonic acid) (Lage et al 1996, L Hales pers comm).

This investigation focused on the suitability of HEPES buffer for the purpose of improving the effectiveness of pH control over the 72 h toxicity test. At 2 mM concentration, HEPES (pKa of 7.2 at 27°C) was found to be a suitable and practical option for pH control and was incorporated in the toxicity test protocol for *Chlorella* sp.

7.2 Determination of the reliability of *Chlorella* sp as a standard test organism

For a species to be considered appropriate for use in toxicity testing, it is important that its response to a toxicant is reproducible. Over the ten-month study, cell division rates of *Chlorella* sp in the controls were highly reproducible and the species sensitivity to Cu and U remained generally similar from week to week. The percentage coefficient of variation over the study period was calculated at <30% and <7% between toxicity tests for Cu and U respectively. There was however, some variability in the 72 h EC_{50} values for Cu at pH 6.5, due in part to the flat concentration-response curve at very low Cu concentrations. On the basis of these results, *Chlorella* sp was considered a fairly reliable species for use in routine testing. The reproducibility of the toxicity test over a longer period, however, is still uncertain.

Using the same methodology as previously outlined, additional Cu and U toxicity tests were carried out several months later to determine the reproducibility of the test over time. Two additional Cu toxicity tests performed at pH 6.5, gave 72 h EC_{50} values of 7.1 ± 1.7 µg Cu L^{-1} and 3.6 ± 0.9 µg Cu L^{-1}. Although these values did not overlap the mean 72 h EC_{50} value of

1.5 ± 1.0 µg Cu L^{-1} originally established for *Chlorella* sp, the species sensitivity to Cu still remained very high. Furthermore, on the basis of the MDEC and BEC_{10} values, less variation was detected between the established results and these additionally derived results. The BEC_{10} values from the additional toxicity tests were 0.9 and 1.4 µg Cu L^{-1} (compared to 0.6 µg Cu L^{-1} derived originally), while the MDEC values were 1.0 and 1.6 µg Cu L^{-1} (compared to 0.7 µg Cu L^{-1} derived originally). This degree of variation was considered acceptable.

Additional toxicity tests were also performed to assess the reproducibility of *Chlorella* sp to U over time. Significant deviation in the reported 72 h EC_{50} was detected for U at pH 6.5. The U toxicity tests were also used as the unbuffered control treatment in the HEPES experiments and the results are shown in section 7.1 (table 20), along with the concentration-response curve (appendix H). The 72 h EC_{50} value at pH 6.5 was 11.3 ± 3.7 µg U L^{-1}. This value was nearly four-times lower than the previous 72 h EC_{50} value for *Chlorella* sp. The increase in the alga's sensitivity to U at pH 6.5 is considered to be largely associated with the distinct change in shape of the concentration-response curve compared to earlier toxicity tests (appendix H). Although *Chlorella* sp has always displayed a non-sigmoidal response in the presence of Cu, a typical sigmoidal response was always detected in the presence of U. The advantage of this sigmoidal-type response is that because the curve is very steep at the intermediate concentration range, a more reproducible EC_{50} value with small 95% confidence intervals is obtained (ie the median percentile growth response occurs around the same toxicant concentration). Instead, the concentration-response curve showed an initial decrease in algal growth at low U concentrations. The shape of this response curve is a problem because small variations in the alga's response to the toxicant between tests may result in marked differences in the EC_{50} value obtained.

The toxicity of U to *Chlorella* sp was also reassessed at pH 5.7. Based on the 72 h EC_{50}, less than a two-fold difference in toxicity was detected compared to the previous toxicity results (ie 72 h EC_{50} value = 100 ± 17 µg U L^{-1} compared to the original value of 78 ± 5 µg U L^{-1}).
Unlike the results obtained at pH 6.5, which detected an increase in the alga's sensitivity to U, the toxicity test at pH 5.7 showed a slight decrease in sensitivity.

7.2.1 Discussion

The investigation to determine the reliability of *Chlorella* sp over time has identified a potential limitation using this species for toxicity assessment. The results indicate that on occasion, the sensitivity of *Chlorella* sp to Cu and U may fluctuate. It should be noted that variations in sensitivity were not a constant and continual factor throughout the period of study. Instead, a transient change in metal sensitivity was detected and this may be largely due to the unusual, often non-sigmoidal, concentration-response curve for this species. Despite the inherent problems associated with this species, it does however possess a number of desirable characteristics and therefore is still recommended as a standard test organism. In particular, the alga's high sensitivity to Cu and U and strong environmental relevance make it a suitable choice for site-specific testing of mine wastewaters in the tropics.

8 Conclusions

This research has yielded several key results:

1 The tropical Australian freshwater alga *Chlorella* sp (new species) was highly sensitive to Cu and U in a synthetic softwater, with 72 h EC_{50} values of 1.5 µg Cu L^{-1} and 44 µg U L^{-1} obtained at pH 6.5. This sensitivity makes it a suitable standard test organism for site-specific testing of mine wastewaters from Ranger uranium mine, Northern Territory.

2 The unusual, often non-sigmoidal, concentration-response curve for *Chlorella* sp reduces the reproducibility of the toxicity test. Although not problematic in the present study, this is an issue which should be acknowledged as a potential limitation associated with using *Chlorella* sp for toxicity assessment.

3 The toxicity of Cu and U to *Chlorella* sp was highly pH-dependent. Metal concentrations needed to inhibit growth by 50% (EC_{50}) increased 18-fold for Cu and up to two-fold for U as the pH decreased from 6.5 to 5.7. Decreased metal toxicity at pH 5.7 was the result of lower concentrations of cell-bound and intracellular Cu or U, possibly due to competitive inhibition of Cu or U with H^+. This pH-dependent toxicity has significant implications for the derivation of appropriate water quality guidelines for protecting freshwater biota in tropical waters.

4 HEPES buffer (2 mM at pH 6.5) was found to be a suitable and practical option for pH control that could be incorporated into the toxicity test protocol for *Chlorella* sp. The results obtained in this study confirmed that HEPES was not toxic, nor did it complex Cu and U.

In summary, the information reported in this study will assist in the development of water quality measures to protect the freshwater ecosystems of Magela Creek and other systems of similar chemistry in tropical Australia. The freshwater algal toxicity test, when used as part of a battery of toxicity tests with other local freshwater organisms, will enable mining companies to assess the ecological risk of a receiving water that may be affected by mine wastewaters. In addition, this study provides relevant toxicity data for Cu and U that may be incorporated into future revisions of the Australian water quality guidelines for the protection of freshwater ecosystems of tropical Australia.

Appendixes

Appendix A Protocol for the algal growth inhibition test using the tropical freshwater green alga *Chlorella* sp

A.1 Principles and applications of the test method

This protocol measures the chronic toxicity of metals to the freshwater green alga *Chlorella* sp isolated from Kakadu National Park, Northern Territory, during a 3 day static exposure.

Exponentially growing cells of *Chlorella* sp are exposed to various concentrations of the toxicant over several generations under defined conditions. Cells are counted each day for 72 h. The growth of the algae exposed to the test substance is compared to the growth of the algae in an appropriate control. A test substance is considered toxic when a statistically significant, concentration-dependent inhibition of algal growth occurs.

A.1.1 Advantages of the test

(a) This species is a local isolate from Kakadu National Park, Northern Territory, making it a useful test organism for assessing site specific impacts of mine wastewaters in this region.

(b) This species is very sensitive to Cu and U in a low nutrient softwater typical of Australian tropical freshwaters.

A.1.2 Limitations of the test

(a) The unusual, often non-sigmoidal, concentration-response curve for this species limits its reproducibility.

A.2 Test organism

A.2.1 Test species

The unicellular freshwater green alga *Chlorella* sp (Chlorophyceae) was isolated from surface water collected within Kakadu National Park, Northern Territory (Padovan 1992) and maintained at the Environmental Research Institute of the Supervising Scientist (***eriss***). The culture was sent to the laboratories of the CSIRO Division of Energy Technology, Sydney.

A.2.2 Stock culture maintenance

The alga is cultured axenically in a modified MBL medium. Ten stock nutrient solutions are prepared in volumetric flasks using analytical grade reagents and Milli-Q water (table 1).

To prepare the liquid growth media, add 0.05 mL of stock solutions 1, 2, 3, 4, 5, 6, 7, 9, and 10, and 0.1 mL of stock solution 8 to 50 mL of Milli-Q water in a 250 mL borosilicate culture flask with a loose fitting glass closure. Autoclave the medium at 121°C and 120 kilopascals for 20-min. Allow to cool overnight for re-equilibration of CO_2. The final concentrations of the macronutrients and micronutrients in the liquid growth medium are shown in table 2.

Aseptically transfer 1 mL of the algal culture to the liquid growth medium in a 250 mL Erlenmeyer flask using a disposable sterile glass pipette. Incubate the culture at 27 ± 1°C on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 75 µmol photons PAR $m^{-2}s^{-1}$). No shaking is necessary. The culture should be renewed every week to ensure a regular supply of exponentially growing cells for the toxicity test.

Routine microscope examination of the algal stock culture, using a phase contrast microscope, should be conducted to ensure good cell morphology and the absence of contaminants.

Table 1 Stock nutrient solutions for the maintenance of *Chlorella* sp (MBL medium)

Stock solutions	Concentration
1. $NaNO_3$	85.24 g L^{-1}
2. $NaHCO_3$	12.6 g L^{-1}
3. K_2HPO_4	8.72 g L^{-1}
4. $CaCl_2$	27.7 g L^{-1}
5. $MgSO_4.7H_2O$	36.97 g L^{-1}
6. $FeCl_3$	0.436 g L^{-1}
7. Na_2EDTA	4.36 g L^{-1}
8. Tris	500 g L^{-1}
9. Trace elements	
$CuSO_4.5H_2O$	9.0 mg L^{-1}
$ZnSO_4.7H_2O$	22.0 mg L^{-1}
$CoCl_2.6H_2O$	10.0 mg L^{-1}
$MnCl_2.4H_2O$	180.0 mg L^{-1}
$Na_2SiO_35H_2O$	7.0 mg L^{-1}
10. Vitamins: Pipette 2.5 mL of a biotin stock (0.05 g/500 mL) and 2.5 mL of a Vitamin B_{12} stock (0.025 g/250 mL) into a 250 mL volumetric containing 0.05 g thiamine. Make up to volume with Milli-Q water.	

Table 2 Final concentration of nutrients in the culture medium for stock culture maintenance of *Chlorella* sp

Nutrient		MBL medium
Major Constituents		**Concentration (mg L^{-1})**
Tris		500
$NaNO_3$		85
$NaHCO_3$		13
K_2HPO_4		8.7
Ca^{2+}		10
Mg^{2+}		6.6
Cl^-		7.9
SO_4		4.8
Na_2EDTA		4.4
Minor Constituents		**Concentration (µg L^{-1})**
Si^{2+}		1.0
Mn^{2+}		50
Zn^{2+}		5.0
Co^{2+}		2.2
Cu^{2+}		2.3
Fe^{3+}		152
Vitamins	– Thiamine	200
	– Biotin	1.0
	– B_{12}	1.0

A.3 Test solution (control/dilution water)

The test water is a synthetic water that simulates the chemical composition of Magela Creek, Northern Territory, during the wet season. Magela Creek is very soft, slightly acidic water and has a low buffering and complexing capacity.

The synthetic water is prepared by adding the quantities specified in table 3 into a 5 L volumetric and filling with Milli-Q water. The water should be adjusted to pH 6.5 with 0.02 M NaOH. The softwater is then filtered through a Millipore HA 0.45 µm membrane filter. An aged and acid-washed 5 L plastic container should be used to store the water at 4°C for no longer than two weeks before use.

Table 3 Inorganic components of synthetic Magela Creek water

Salt	Stock solution ($g\ L^{-1}$)	Amount added to 5 L of Milli-Q water (µL)	Total concentration ($\mu g\ L^{-1}$)
$MgSO_4.\ 7H_2O$	138	222	600 Mg^{2+}
			2370 SO_4^{2-}
$CaCl_2.\ 2H_2O$	37.1	222	449 Ca^{2+}
			796 Cl^-
$NaHCO_3$	81.5	222	991 Na^+
			2630 HCO_3^-
KCl	16.0	222	372 K^+
			338 Cl^-
$FeCl_3.\ 6H_2O$	9.79	222	89.9 Fe^{3+}
			171 Cl^-
$Al_2(SO_4)_3.\ 18H_2O$	19.5	222	70.2 Al^{3+}
			375 SO_4^{2-}
$MnSO_4.\ H_2O$	1.34	111	9.68 Mn^{2+}
			16.9 SO_4^{2-}
$CuSO_4.\ 5H_2O$	0.124	111	0.701 Cu^{2+}
			1.06 SO_4^{2-}
$ZnSO_4.\ 7H_2O$	0.139	111	0.702 Zn^{2-}
			1.03 SO_4^{2-}
$UO_2SO_4.\ 3H_2O$	0.0077	111	0.11 UO_2^{2+}
			0.039 SO_4^{2-}

A.4 Toxicity test facility and equipment

The algal toxicity test should be conducted in a facility with controlled temperature and lighting, such as an environmental cabinet or growth room. Test conditions should be uniform throughout the chamber and identical to the culturing facility.

All instruments for routine measurements of the basic chemical, physical and biological variables must be maintained and regularly calibrated. Any equipment that makes contact with the test organism, media, control water or test solutions must be made of chemically inert materials (eg glass, polyethylene) and be thoroughly cleaned before use. Tables 4 and 5 list the consumable and non-consumable equipment necessary to conduct the toxicity test.

Table 4 Non-consumable equipment required for *Chlorella* sp toxicity test

- haemocytometer or electronic particle counter for algal cell enumeration
- environmental chamber or growth room with light boxes and temperature recorder
- Milli-Q water purification system or equivalent
- microscope with phase contrast providing 200 × magnification
- centrifuge – 4 × 30 mL capacity with swing out buckets
- refrigerator
- vortex mixer
- glass tissue homogeniser (hand-held – 15 mL) with teflon pestle
- glass centrifuge tubes – 30 mL capacity
- borosilicate glass 200 mL Erlenmeyer flasks with loose glass caps
- glass volumetric flasks
- glass graduated measuring cylinders
- centrifuge tube racks (perspex or equivalent)
- analytical balance and weighing spatula
- pH meter
- light meter
- filter apparatus: 47 mm glass filter holder, 1 L flask, vacuum pump and tubing
- magnetic stirrer and stirrer bars
- automatic adjustable pipettes (5 µL to 5 mL)
- polyethylene wash bottles and storage containers (1 L to 10 L)

Table 5 Consumable equipment required for *Chlorella* sp toxicity test

- disposable sterile glass graduated 2 mL pipettes
- disposable glass Pasteur pipettes
- disposable microlitre pipette tips
- membrane filters – 0.45 µm pore size
- Parafilm or equivalent laboratory sealing film
- weighing dishes
- haemocytometer cover glasses
- disposable plastic counting cups for the electronic particle counter
- filtered seawater for electronic particle counting
- chemicals and reagents

A.5 Glassware washing procedure

All reusable glassware which has been in contact with the metals must be washed with a non-phosphate detergent (eg Decon). After rinsing with de-ionised water, the glassware should then be soaked overnight in 10% nitric acid (AR) and rinsed five times with de-ionised water and five times with Milli-Q water.

Erlenmeyer flasks (250 mL) used exclusively in the toxicity tests should be soaked and washed in the same manner as described above. These flasks should then be coated with a

silanising solution such as Coatasil (Ajax), dried in the fume cupboard for 24 h and acid-washed again immediately before use. This silanising step is essential to reduce metal adsorption to the walls of the glass flasks, thereby reducing their availability and possibly toxicity to the algae during the test. Silanisation of the flasks should be redone monthly as continual washing gradually removes the surface coating.

At the completion of the toxicity test, the flasks should be rinsed with de-ionised water followed by an acetone rinse, before being acid-washed as above.

Glassware used only for culturing the alga requires acid-washing in 10% nitric acid only as above. This glassware should not be used for toxicity testing.

A.6 Toxicity test procedure

A summary of the test procedure conditions is shown in table 6.

Table 6 Summary of recommended toxicity test conditions for the tropical freshwater alga (*Chlorella* sp) growth inhibition test

1. Test type	Static
2. Temperature	27°C ± 1°C
3. Light quality	Cool white fluorescent lighting
4. Light intensity	115–125 µmol photons PAR $m^{-2}s^{-1}$
5. Photoperiod	12 hour light: 12 hour dark
6. Test chamber size	250 mL
7. Test solution volume	50 mL
8. Renewal of test solutions	None
9. Age of test organisms	4–5 d
10. Initial cell density in test chambers	$2–4 \times 10^4$ cells mL^{-1}
11. No. of replicate chambers/concentration	3
12. Shaking rate	Twice daily by hand
13. Test medium	Synthetic softwater + 15 mg L^{-1} NO_3
	+ 0.15 mg L^{-1} PO_4 + 2 mM HEPES buffer (pH 6.5)
14. Metal concentrations	Minimum of 5 and a control
15. Test duration	72 h
16. Test endpoint	Growth (cell division)
17. Test acceptability	Control cell division rates 1.6 ± 0.3 doublings day^{-1}

A.6.1 Preparation of the algal inoculum

The algal inoculum must be prepared no more than 2–3 h before incubation in the toxicity test. The inoculum is composed of exponentially growing *Chlorella* cells harvested from a 4–5 d old stock culture.

The algal cells are centrifuged in 30 mL glass centrifuge tubes at 2500 revolutions per minute (rpm) at 20°C in a Joun CR4.11 refrigerated centrifuge for 7 min. The nutrient medium (supernatant) is decanted and the cell pellet re-suspended in about 30 mL of Milli-Q water by gentle vortexing. The centrifugation and washing procedure is repeated three times to remove the high nutrient culture medium, which would otherwise ameliorate toxicity due to its ability

to strongly complex trace metals (Stauber & Florence 1989). The cell pellet is finally re-suspended in about 15 mL of Milli-Q water.

The re-suspended cells are gently homogenised in a hand-held glass tissue grinder with teflon pestle to disaggregate cell clumps. An aliquot of cell suspension (inoculum 0.1 mL) is added to a 250 mL flask containing 50 mL of synthetic softwater. The cell density in this flask is determined microscopically using a haemocytometer. The desired inoculum is calculated to give a final cell density of $2–4 \times 10^4$ cells mL^{-1} in the test flask.

A.6.2 Preparation of test solutions

Triplicate controls are prepared by dispensing 55 mL of filtered synthetic water into clean silanised 250 mL glass Erlenmeyer flasks. At least five toxicant concentrations are prepared by using the same filtered synthetic water as was used for the preparation of the control flasks. Test concentrations of the metals should be in a geometric series (ie 2.5, 5, 10, 20 40 µg L^{-1} etc). Neutralisation should be carried out by the addition of appropriate amounts of dilute NaOH if the pH of the medium changes >0.1 unit with the addition of the metals. To all flasks add 0.5 mL of 26 mM sodium nitrate (15 mg NO_3 L^{-1}), 0.05 mL of 1.3 mM potassium dihydrogen phosphate (0.15 mg PO_4 L^{-1}) and 0.22 mL of 0.5 M HEPES buffer (*N*-2 hydroxyethylpiperazine-*N*'-2ethanesulfonic acid) to 54.5 mL of filtered synthetic softwater. Stauber et al (1994) found this concentration of nitrate and phosphate to be the only nutrients required to maintain exponential growth over a 3 d test for the temperate freshwater alga *Chlorella protothecoides*.

A 5 mL sub-sample from each flask is then transferred into an acid-washed polyethylene container. Triplicate sub-samples are combined to give a volume of 15 mL which is acidified with 30 µL concentrated nitric acid. Metals should then be analysed by the appropriate technique.

Test flasks are inoculated with $2–4 \times 10^4$ cells mL^{-1} of prewashed *Chlorella* cells. To one flask, no algae is added. This serves as a background correction for the automatic particle counts. Each test flask is incubated at 27 ± 1°C on a 12:12 h light/dark cycle (Philips TL 40W cool white fluorescent lighting, 125 µmol photons PAR $m^{-2}s^{-1}$).

As standard practice, all assay flasks are to be shaken twice daily by hand to avoid gas limitation. This consists of swirling the solution approximately six times in the clockwise direction and six times in the anti-clockwise direction. On days 0 and 3 of the toxicity test, the pH should be measured in one replicate flask from each test treatment using a pH meter.

A.6.3 Algal cell counts

Cell density in each flask is determined daily for three days. Cell counts can be performed using either an automatic particle counter, such as a Coulter Multisizer II, or manually using a haemocytometer and a microscope.

Electronic cell enumeration

A Coulter Multisizer II Particle Analyser with a 70 µm aperture is used in the narrow mode with window settings ranging from 1.45–9.36. Before counting, the test flasks must be well mixed by swirling the solution six times in a clockwise direction and six times in an anti-clockwise direction. Immediately take a 2.5 mL aliquot of cells from each flask and dilute to 10 mL with 0.45 µm filtered seawater. This dilution in seawater is necessary to provide sufficient electrolyte for electronic particle counting. Samples should be homogenised gently in a tissue grinder to break cell clumps prior to counting. Pour the sample directly into a plastic counting cup and count the algae with the particle counter. Four 100 µL aliquots should be counted for each flask and the mean count ± 2 standard deviations (SD) determined.

To avoid the problem of two cells being counted as one, cell suspensions should be diluted so that the coincidence correction is <35%.

Each day, a background count (1:4 dilution with seawater) from a test flask containing no algal cells is determined. This 'Coulter blank', arising from small particles other than algal cells, is then subtracted from the mean algal cell count for each flask.

Manual cell enumeration

Algal cells may be counted using a phase contrast microscope and a haemocytometer. Although this method is less precise than the electronic counting method, it does permit the direct examination of the cell morphology and is necessary on day 0 when cell densities are low.

Sub-samples from each flask (1–2 mL) are taken and homogenised in a tissue grinder to break cell clumps. No dilution with seawater or background count correction is required. At least 200 individual cells should be counted per sample, to maintain counting errors of <20%.

A.6.4 Test endpoints and calculations

The growth rate (cell division rate) for each flask over 72 h is calculated using regression analysis. A regression line is fitted to a plot of the $\log_{10}$ cell density vs time (h). The slope of the regression line for each flask is equivalent to the cell division rate per hour (μ) for each treatment. Daily doubling times can be calculated by multiplying this value $\mu \times 24 \times 3.32$ (constant). This is acceptable if the control growth rates are 1.6 ± 0.3 doublings day^{-1}. Growth rates of the treated flasks are presented as a percentage of the control growth rate. A calibration curve can be then fitted to the data by plotting the percentage control growth rates vs the measured toxicant concentrations.

The endpoints of the algal growth inhibition test are the 72 h EC_{50}, BEC_{10} and MDEC (or NOEC and LOEC). The 72 h EC_{50} is the effective toxicant concentration which gives 50% reduction in algal growth rate over 72 h compared to the controls. This is calculated using the Trimmed Spearman-Karber analysis (Hamilton et al 1977) or other appropriate statistics software. Ninety-five percent confidence limits should also be quoted. The lower the EC_{50} value, the more toxic is the sample.

The 10% bounded effect concentration (BEC_{10}), an alternative to the NOEC, can be estimated from the concentration-response data using the approach described by Hoekstra and van Ewijk (1993). The minimum detectable effect concentration (MDEC), an alternative to the LOEC, can be estimated using the approach described by Ahsanullah and Williams (1991).

Appendix B Sterility check of algal cultures

B.1 Freshwater peptone yeast extract agar (PYEA) plates

To 300 mL of Milli-Q water add:

0.3 g peptone water

0.3 g yeast extract

6 g bacteriological agar

Mix well, autoclave for 20 min at 126°C in a schott bottles. Cool until the bottle is just able to be held. Pour into sterile disposable petri dishes. Cool overnight and store plates upside down in a sealed plastic bag at 4°C.

Appendix C Summary data for *Chlorella* sp 12 toxicity test and optimisation tests using *Chlorella* sp

Table 1 Toxicity of Cu to *Chlorella* sp 12 (data from fig 8)

Nominal Cu ($\mu g\ L^{-1}$)	Measured Cu ($\mu g\ L^{-1}$) [a]	Mean cell division rate over 72 h		CV (%)
		(doublings d^{-1})	% of control	
0	Control	1.35	100	3.7
2.5	2.5	1.32	98	4.8
5.1	5.1	1.27	95	3.2
7.5	7.5 [b]	0.93	69	10
10	10 [b]	0.13	10	100
20	19 [b]	0.00	0.0	173
40	39 [b]	0.02	1.0	173

a Cu concentration measured by GFAAS on day 0 of the toxicity test

b The mean cell division rate at these Cu concentrations were significantly different to the mean of the controls at $P \leq 0.05$ based on Dunnett's test

Table 2 Control cell division rates of *Chlorella* sp at varying nitrate and phosphate concentrations

Nitrate ($mg\ L^{-1}$)	Phosphate ($mg\ L^{-1}$)	Mean cell division rate (doublings d^{-1})	pH	
			day 0	day 3
1.0	0.15	1.35	6.40	6.66
2.5	0.15	1.23	6.55	6.80
5.0	0.15	1.44	6.56	6.57
10	0.15	1.44	6.56	7.19
15	0.15	1.55	6.55	7.40
20	0.15	1.33	5.54	7.13
15	<0.018	0.94	6.24	6.72
15	0.15	1.69	6.20	7.03
15	0.30	1.77	6.41	7.06
15	0.75	1.52	6.31	7.02
15	1.50	1.49	6.29	7.00

Table 3 Daily pH measurements from optimisation test on the effect of pH adjustment on algal growth rates (pH 5.7 and 6.0)

Treatment	day 0	day 1	day 2	day 3
pH 5.7 unadjusted	5.67	5.90	6.22	6.78
pH 5.7 unadjusted	5.67	5.98	6.43	6.95
pH 5.7 unadjusted	5.68	5.95	6.39	6.86
pH 5.7 adjusted	5.69*	6.02	6.22	6.79
pH 5.7 adjusted	5.74*	6.01	6.23	6.74
pH 5.7 adjusted	5.73*	6.02	6.20	6.73
pH 6.0 unadjusted	5.96	6.15	6.37	7.06
pH 6.0 unadjusted	5.98	6.21	6.39	7.18
pH 6.0 unadjusted	5.98	6.17	6.39	7.13
pH 6.0 adjusted	6.00*	6.09*	6.35	6.99
pH 6.0 adjusted	6.01*	6.07*	6.35	6.87
pH 6.0 adjusted	6.01*	6.06*	6.33	6.81

* indicates that flasks did not have to be adjusted (ie no addition of HCl or NaOH) for pH
Flasks 4–6 adjusted to pH 5.7 ± 0.1 daily. Flask 10–12 adjusted to pH 6.0 ± 0.1 daily

Table 4 Daily pH measurements from growth rate toxicity test at pH 5.0, 5.7, 6.5 and 7.0

Treatment	day 1 (M)	day 1 (A)	day 2 (M)	day 2 (A)	day 3 (M)
pH 5.0	4.97	5.32	5.10	6.23	4.98
pH 5.0	5.08	5.27	5.09	6.19	5.06
pH 5.0	5.10	5.25	5.05	6.05	5.06
pH 5.7	5.78	6.29	5.95	6.80	6.20
pH 5.7	5.98	6.26	5.94	6.92	6.30
pH 5.7	5.92	6.30	6.07	6.97	6.38
pH 6.5	6.54	6.85	6.80	7.48	7.40
pH 6.5	6.52	6.62	6.80	7.67	7.48
pH 6.5	6.59	6.72	6.85	7.72	7.54
pH 7.0	6.82	6.97	7.27	7.32	7.51
pH 7.0	6.82	6.94	7.23	7.38	7.72
pH 7.0	6.82	6.94	7.17	7.38	7.72

M = morning A = afternoon

Appendix D Concentration-response data for copper and uranium toxicity tests using *Chlorella* sp

Table 1 Toxicity of Cu to *Chlorella* sp (1st definitive toxicity test, pH 5.7)

Nominal Cu concentration	Measured Cu concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1}) [a]	(doublings d^{-1})	% of control	
0.7 (Control)	0.5 (Control)	1.46	100	1
2.5	2.6 [b]	1.18	81	6
5.0	4.6 [b]	1.25	85	3
10	9.0 [b]	0.91	62	2
20	19 [b]	0.74	50	3
40	38 [b]	0.58	40	1
80	83 [b]	0.60	41	4
160	169 [b]	0.63	43	5

a Cu concentration measured on day 0 of the toxicity test
b The mean cell division rate at these Cu concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 2 Toxicity of Cu to *Chlorella* sp (2nd definitive toxicity test, pH 5.7)

Nominal Cu concentration	Measured Cu concentration	Mean cell division rate		CV (%)[d]
(µg L^{-1})	(µg L^{-1}) [a]	(doublings d^{-1})	% of control	
0.7 (Control)	0.5 (Control)	1.34	100	4
2.5	2.5 [c]	0.98	73	2
5.0	4.8 [b]	0.85	63	6
10	9.2 [b]	0.81	60	4
20	20 [c]	0.72	54	4
40	40 [c]	0.65	49	2
80	80 [c]	0.57	42	2

a Cu concentration measured on day 0 of the toxicity test
b The mean cell division rate at these Cu concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Nominal concentrations only
d Percentage coefficient of variation around the mean cell division rate

Table 3 Toxicity of Cu to *Chlorella* sp (3rd definitive toxicity test, pH 5.7)

Nominal Cu concentration	Measured Cu concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.7 (Control)	0.5 (Control)	1.29	100	3
1.5	1.4 [b]	1.25	96	0
2.5	2.3 [b]	1.00	78	7
5.0	4.7 [b]	0.83	64	13
10	9.0 [b]	0.78	61	4
20	18 [b]	0.69	54	1
40	38 [b]	0.69	53	4
80	77 [b]	0.66	51	5
160	169 [b]	0.61	48	5
320	306 [b]	0.40	31	13
640	611 [b]	0.00	5	141

a Cu concentration measured on day 0 of the toxicity test
b The mean cell division rate at these Cu concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 4 Toxicity of Cu to *Chlorella* sp (1st definitive toxicity test, pH 6.5)

Nominal Cu concentration	Measured Cu concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.7 (Control)	0.6 (Control)	1.72	100	7
2.5	2.3 [b]	0.83	48	3
5.0	4.5 [b]	0.81	47	1
10	8.1 [b]	0.79	46	6
20	18 [b]	0.62	36	5
40	43 [b]	0.58	34	0
80	74 [b]	0.53	31	9
160	152 [b]	0.10	6	26

a Cu concentration measured on day 0 of the toxicity test
b The mean cell division rate at these Cu concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 5 Toxicity of Cu to *Chlorella* sp (2nd definitive toxicity test, pH 6.5)

Nominal Cu concentration	Measured Cu concentration	Mean cell division rate		CV (%)[d]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.7 (Control)	0.5 (Control)	1.67	100	3
2.5	2.5 [bc]	0.79	47	5
5.0	4.6 [b]	0.60	36	2
10	8.9 [b]	0.51	31	2
20	20 [bc]	0.43	26	2
40	40 [bc]	0.37	22	30
80	80 [bc]	0.36	21	11

a Cu concentration measured on day 0 of the toxicity test
b The mean cell division rate at these Cu concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Nominal concentrations only
d Percentage coefficient of variation around the mean cell division rate

Table 6 Toxicity of Cu to *Chlorella* sp (3rd definitive toxicity test, pH 6.5)

Nominal Cu concentration	Measured Cu concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.7 (Control)	0.6 (Control)	1.71	100	3
1.3	1.2 [b]	1.17	53	14
2.5	2.1 [b]	0.68	40	16
5.0	4.2 [b]	0.57	33	9
10	9.0 [b]	0.55	32	19
20	18 [b]	0.46	27	5
40	41 [b]	0.56	33	5
80	77 [b]	0.41	24	33
160	168 [b]	0.15	9	14

a Cu concentration measured on day 0 of the toxicity test
b The mean cell division rate at these Cu concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 7 Toxicity of U to *Chlorella* sp (1st definitive toxicity test, pH 5.7)

Nominal U concentration	Measured U concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.1 (Control)	<1 (Control)	1.00	100	14
10	8.0 [b]	0.91	96	6
20	17 [b]	0.95	100	10
40	33 [b]	0.88	92	7
80	65 [b]	0.64	67	3
160	127 [b]	0.00	0.0	0

a U concentration measured on day 0 of the toxicity test
b The mean cell division rate at these U concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 8 Toxicity of U to *Chlorella* sp (2nd definitive toxicity test, pH 5.7)

Nominal U concentration	Measured U concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.1 (Control)	<1 (Control)	1.11	100	10
10	8.0 [b]	1.08	97	12
20	16 [b]	1.01	91	11
40	34 [b]	0.97	87	2
80	67 [b]	0.90	81	8
160	134 [b]	0.09	8	173
320	265 [b]	0.00	0	0

a U concentration measured on day 0 of the toxicity test
b The mean cell division rate at these U concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 9 Toxicity of U to *Chlorella* sp (3rd definitive toxicity test, pH 5.7)

Nominal U concentration	Measured U concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.1 (Control)	<1 (Control)	1.12	100	6
20	16 [b]	1.07	96	10
80	64 [b]	0.90	81	16
100	84 [b]	0.57	51	2
120	100 [b]	0.55	49	8
140	118 [b]	0.08	8	7
160	130 [b]	0.08	8	87
250	217 [b]	0.00	0	0

a U concentration measured on day 0 of the toxicity test
b The mean cell division rate at these U concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 10 Toxicity of U to *Chlorella* sp (1st definitive toxicity test, pH 6.5)

Nominal U concentration	Measured U concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.1	<1	1.62	100	1
20	18 [b]	1.13	70	8
40	35 [b]	1.01	62	6
80	70 [b]	0.66	40	26
120	104 [b]	0.30	18	16
160	138 [b]	0.07	5.0	102
320	271 [b]	0.00	0.0	0

a U concentration measured on day 0 of the toxicity test
b The mean cell division rate at these U concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 11 Toxicity of U to *Chlorella* sp (2nd definitive toxicity test, pH 6.5)

Nominal U concentration	Measured U concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.1	<1	1.56	100	1
5.0	4.0 [b]	1.66	105	4
10	9.0 [b]	1.57	101	2
20	18 [b]	1.26	81	8
40	35 [b]	1.06	68	6
80	69 [b]	0.74	47	13
120	103 [b]	0.16	10	27
160	140 [b]	0.00	0.0	0
240	201[b]	0.00	0.0	173

a U concentration measured on day 0 of the toxicity test
b The mean cell division rate at these U concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Table 12 Toxicity of U to *Chlorella* sp (3rd definitive toxicity test, pH 6.5)

Nominal U concentration	Measured U concentration	Mean cell division rate		CV (%)[c]
(µg L^{-1})	(µg L^{-1})[a]	(doublings d^{-1})	% of control	
0.1	<1	1.62	100	4
5.0	4.0 [b]	1.63	101	2
10	8.0 [b]	1.58	98	1
20	17 [b]	1.24	77	14
40	34 [b]	1.12	69	2
80	69 [b]	0.80	49	14
120	101 [b]	0.24	15	16
160	134 [b]	0.10	6.0	51
240	205 [b]	0.00	1.0	125

a U concentration measured on day 0 of the toxicity test
b The mean cell division rate at these U concentrations was significantly different (P at α = 0.05) to the mean of the controls based on Dunnett's test
c Percentage coefficient of variation around the mean cell division rate

Appendix E Summary of copper and uranium toxicity endpoints (48 h and 72 h data)

Toxicity test	48 h Data			72 h data		
	EC_{25}	EC_{50} (µg L^{-1})	EC_{75}	EC_{25}	EC_{50} (µg L^{-1})	EC_{75}
Cu at pH 5.7						
1st definitive Cu	1.0 (0.1–2.9)	80 (40–293)	>1000	4.2 (0.3–11)	20 (12–32)	350 (100–>1000)
2nd definitive Cu	21 (13–35)	292 (122–1814)	>1000	1.4 (0.3–3.0)	32 (17–63)	714 (219–>1000)
3rd definitive Cu	2.0 (0.1–6.4)	40 (29–53)	>1000	4.6 (0.9–11)	38 (30–46)	358 (134–>1000)
Pooled data	–	–	–	4.9 (1.3–11)	35 (28–42)	269 (116–>1000)
Cu at pH 6.5						
1st definitive Cu	0.4 (0.1–2.1)	3.4 (0.9–13)	64 (34–81)	0.2 (0.0–1.6)	3.6 (0.1–10)	57 (21–>1000)
2nd definitive Cu	1.2 (0.4–2.1)	12 (7.5–20)	85 (52–191)	0.04 (0.0–1.0)	1.1 (0.1–2.5)	29 (17–74)
3rd definitive Cu	0.3 (0.0–2.1)	8.9 (4.6–17)	128 (28–>1000)	0.03 (0.0–0.1)	1.4 (1.0–1.9)	31 (19–65)
Pooled data	0.5 (0.0–2.3)	8.4 (4.4–16)	71 (24–>1000)	0.08 (0.0–0.2)	1.5 (0.8–2.8)	35 (22–65)
U at pH 5.7						
1st definitive Cu	*	48 (44–53)	*	*	70 (65–75)	*
2nd definitive Cu	*	64 (57–71)	*	*	77 (70–85)	*
3rd definitive Cu	*	52 (47–58)	*	*	79 (73–86)	*
Pooled data	*	–	*	*	78 (73–83)	*
U at pH 6.5						
1st definitive Cu	*	29 (25–33)	*	*	48 (39–60)	*
2nd definitive Cu	*	35 (32–38)	*	*	45 (40–50)	*
3rd definitive Cu	*	49 (43–55)	*	*	46 (41–51)	*
Pooled data	*	–	*	*	44 (39–49)	*

EC_{50} estimated by Trimmed Spearman-Karber Analysis. EC_{25} and EC_{75} estimated by Maximum Likelihood-Probit Analysis. Values in brackets represents 95% CI. – indicates pooled value not determined due to non-overlapping 95% CI between individual test runs.* EC_{25} and EC_{75} not calculated for U data as sigmoidal-type response was obtained making the EC_{50} a more appropriate endpoint

Appendix F Chemical analysis of synthetic water

Table 1 Measured concentration of inorganic components of synthetic Magela Creek water

Parameter	Unit	Mean Value	S.E.	n
pH	–	5.7 ± 0.5 6.5 ± 0.5	–	–
Temp.	°C	27	–	–
Na	mg L^{-1}	6.80[a]	0.22	9
K	mg L^{-1}	0.46	0.02	9
Ca	mg L^{-1}	0.47	0.01	9
Mg	mg L^{-1}	0.67	0.02	9
Cl	mg L^{-1}	1.35	0.03	4
SO_4	mg L^{-1}	2.85	0.06	4
Fe	µg L^{-1}	<50[b]	–	9
Al	µg L^{-1}	34	2.42	9
Mn	µg L^{-1}	10	0.00	9
U	µg L^{-1}	<1[b]	–	6
Cu	µg L^{-1}	0.6	0.07	6

a Measured concentration considerably higher than nominal due to Na input from the addition of NO_3 as $NaNO_3$ (algal nutrient)

b Practical quantitation limit

Table 2 Measured concentration of algal nutrients added to test water

Parameter	Unit	Mean Value	S.E.	n
NO_3	mg L^{-1}	19.3	0.40	6
PO_4	mg L^{-1}	0.16	0.01	6

Appendix G Additional results from intracellular and extracellular copper/uranium experiments

Table 1 Mass balance of Cu fractions at pH 5.7 and pH 6.5

Cu fraction	[Cu] μgL^{-1}	Mean (%) ± one SD	
		pH 5.7	pH 6.5
Dissolved Cu	10	46 ± 9	32 ± 14
	80	74 ± 2	63 ± 3
	160	–	71 ± 0.2
	640	92 ± 1	–
Flask bound Cu	10	37 ± 9	39 ± 13
	80	18 ± 0.1	25 ± 3
	160	–	24 ± 0.9
	640	6 ± 0.9	–
Intracellular Cu	10	10 ± 0.9	16 ± 1
	80	3 ± 0.1	5 ± 0.7
	160	–	2 ± 0.2
	640	1 ± 0.1	–
Extracellular Cu	10	7 ± 0.7	13 ± 7
	80	5 ± 2	7 ± 3
	160	–	3 ± 0.7
	640	2 ± 0.9	–

Table 2 Mass balance of U fractions at pH 5.7 and pH 6.5

U fraction	[U] μg L^{-1}	Mean (%) ± one SD	
		pH 5.7	pH 6.5
Dissolved U	40	38 ± 9	31 ± 9
	120	67 ± 5	49 ± 15
	250	71 ± 9	71 ± 8
Flask bound U	40	41 ± 7	40 ± 5
	120	24 ± 3	38 ± 14
	250	21 ± 7	19 ± 8
Intracellular U	40	9 ± 2	9 ± 2
	120	4 ± 0.2	4 ± 0.2
	250	7 ± 5	3 ± 0.5
Extracellular U	40	12 ± 7	20 ± 12
	120	6 ± 2	8 ± 2
	250	5 ± 0.7	7 ± 2

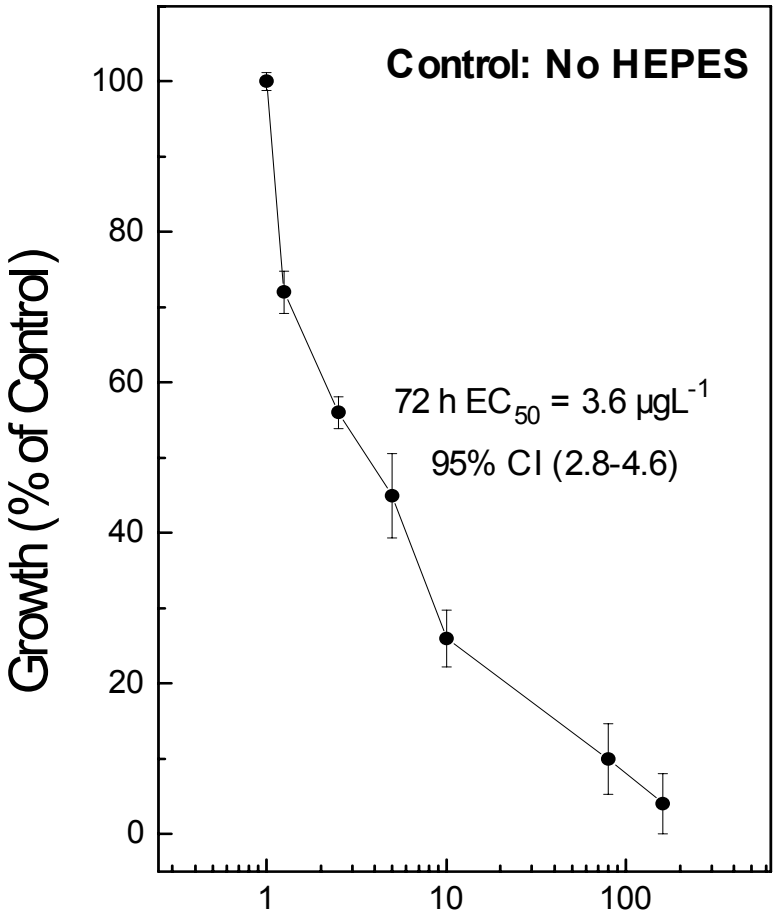
Table 3 Intracellular and extracellular U in *Chlorella* sp in relation to pH (on a cell surface area basis)

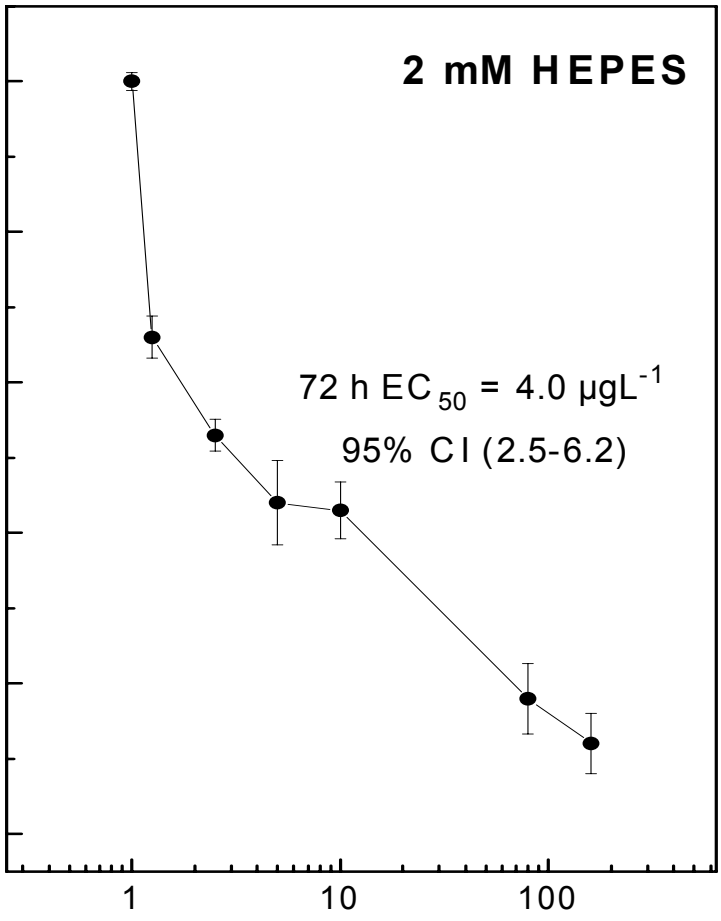
Ph 5.7				Ph 6.5			
[U] µg L^{-1}		Intra-U	Extra-U	[U] µg L^{-1}		Intra-U	Extra-U
		(x10 $^{-6}$ng/µm^2)				(x10 $^{-6}$ng/µm^2)	
40	Mean	0.85	1.09	40	Mean	1.17	2.75
	SD	0.19	0.54		SD	0.38	1.67
120	Mean	1.47	2.43	120	Mean	2.07	24.43
	SD	0.15	0.85		SD	0.17	0.70

Table 4 Mean cell surface area of Chlorella sp cells exposed to U at pH 5.7 and pH 6.5

Treatment		Algal cell surface area (µm^2)	
		pH 5.7	pH 6.5
Control	Mean	15.7	15.1
	SD	0.47	0.07
40 µg U L^{-1}	Mean	19.8	29.7
	SD	0.10	15.2
120 µg U L^{-1}	Mean	47.9	44.0
	SD	1.24	2.17

Appendix H Influence of HEPES buffer on cell division rates of *Chlorella* sp exposed to copper and uranium


Control: No HEPES
72 h EC50 = 3.6 µgL-1
95% CI (2.8-4.6)
Growth (% of Control)
100
80
60
40
20
0
1
10
100


2 mM HEPES
72 h EC50 = 4.0 µgL-1
95% CI (2.5-6.2)
1
10
100

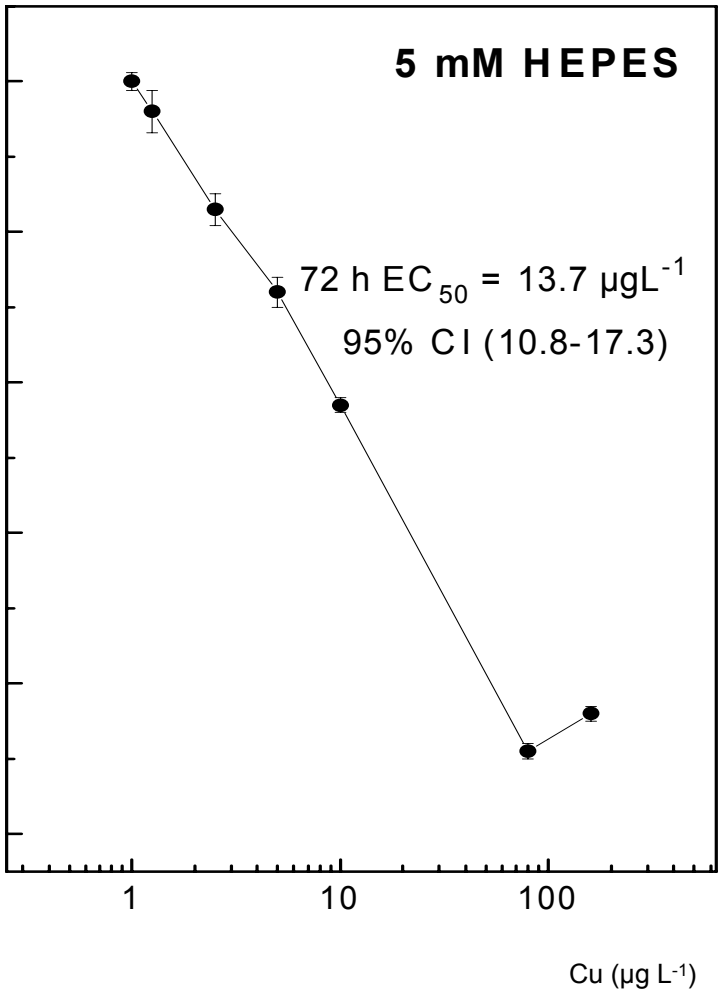


Figure 1 Growth inhibition of *Chlorella* sp exposed to Cu in the presence and absence of HEPES buffer (2 mM and 5 mM)

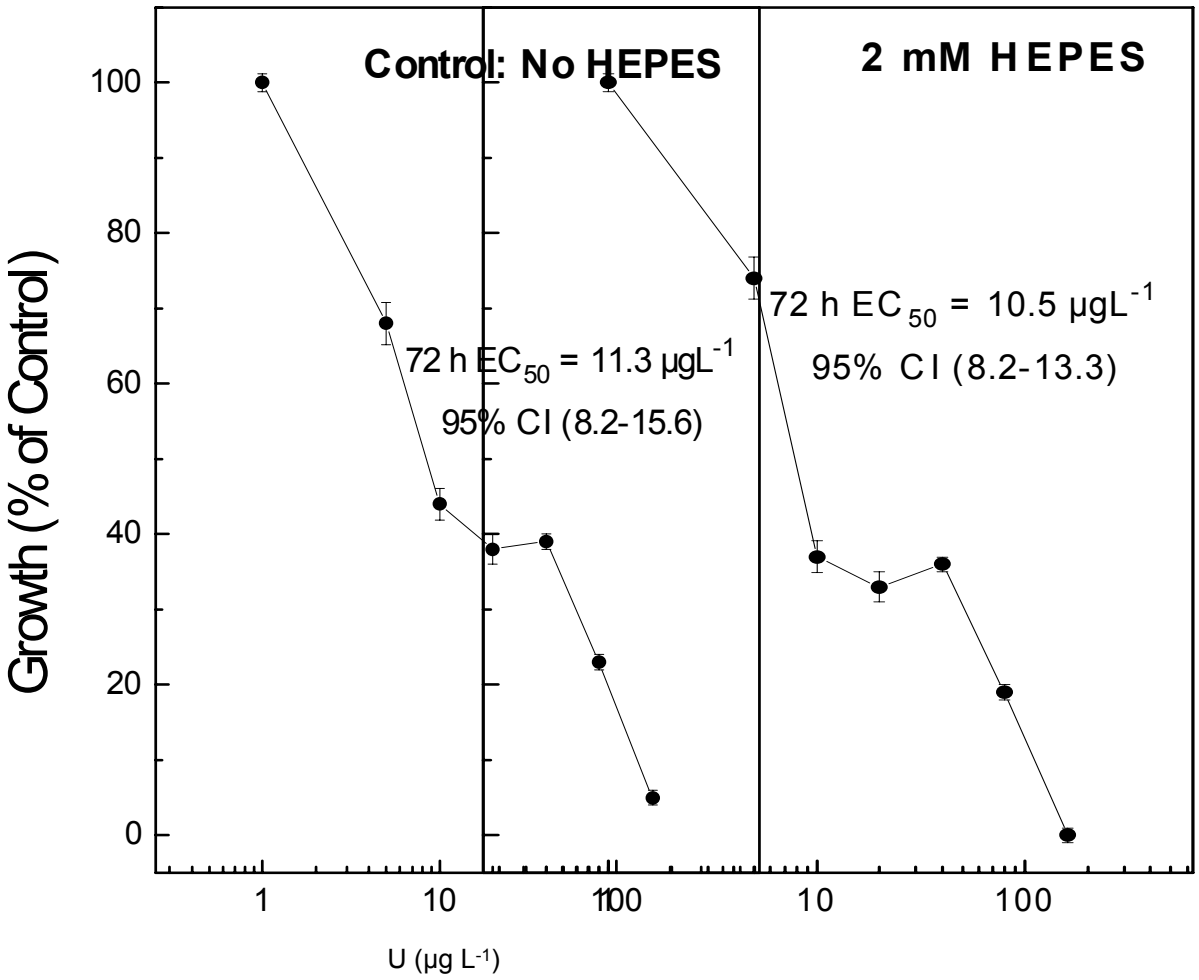


Figure 2 Growth inhibition of *Chlorella* sp exposed to U in the presence and absence of HEPES buffer (2 mM)

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