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# MOUNT LYELL REMEDIATION

## Toxicity assessment of waters from Macquarie Harbour, western Tasmania, using algae, invertebrates and fish

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A joint program between the Supervising Scientist and the Department of Environment and Land Management, Tasmania.

This report describes research that is part of the Mt Lyell Remediation Research and Demonstration Program, a joint program between the Supervising Scientist and the Department of Environment and Land Management, Tasmania.



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# Executive summary

The 100-year operation of the Mount Lyell Mining and Railway Company Limited's copper mine in Queenstown, Tasmania, has resulted in the deposition of over 100 million cubic metres of mine tailings, smelter slag and topsoil into the King River and Macquarie Harbour. Following cessation of tailings discharge into the rivers in December 1994, the Commonwealth and Tasmanian governments are undertaking the Mount Lyell Remediation, Research and Demonstration Program to assess the environmental impact of metal release from the mine and smelter, as part of the development of a remediation strategy. In particular, an assessment of the potential biological impact of elevated copper levels in Macquarie Harbour waters is required.

The objective of this project was to conduct toxicity tests to determine the concentration of copper which can be tolerated in Macquarie Harbour waters without causing detriment to aquatic life. Sensitive sub-lethal tests including phytoplankton growth and enzyme inhibition, amphipod growth inhibition and osmoregulatory and histopathological responses of juvenile flounder were used to determine the toxicity of mid-salinity Macquarie Harbour waters. In addition, a preliminary risk assessment of the potential for toxic effects from copper in Macquarie Harbour is provided, based on values of toxicity in marine and estuarine waters reported in the literature.

Total dissolved copper in Macquarie Harbour waters collected in October and December 1995 for use in the bioassays, ranged from 10 to 42 µg Cu/L, with the highest concentrations immediately below the outflow of the King River. Dissolved copper concentrations exceeded the complexing capacity of the dissolved organic matter in all samples. Electrochemical methods showed that significant amounts of dissolved copper in the Macquarie Harbour waters were present in labile forms (6–24 µg Cu/L) and potentially bioavailable.

Although chemical measurements of copper in the Macquarie Harbour waters, together with the preliminary risk assessment suggested that copper was potentially bioavailable, toxicity testing of mid-salinity Macquarie Harbour waters revealed that there were no significant effects on algal growth, amphipod and juvenile flounder survival, or osmoregulation and copper accumulation in flounder. Of these endpoints, only algal growth inhibition and copper accumulation in flounder were sensitive enough to detect potential effects at copper concentrations found in Macquarie Harbour.

The growth inhibition bioassay with the marine alga *Nitzschia closterium* was particularly sensitive to copper, with ionic copper concentrations as low as 5 µg/L causing a significant reduction in algal cell division rate over 72 h. However, in general, no toxicity of filtered or unfiltered Macquarie Harbour waters was observed, suggesting that copper in these waters was not present in bioavailable forms. There was, however, significant bioavailable copper to cause inhibition of enzyme activity (ß-D-galactosidase) in the marine alga *Dunaliella tertiolecta* and this bioavailable copper was weakly correlated with labile copper in the Macquarie Harbour waters measured using anodic stripping voltammetry. It is possible that colloidal iron, manganese and aluminium oxides/hydroxides bind copper at the algal cell surface, preventing its uptake into the cell. Enzyme inhibition may occur at the cell membrane, whereas growth is not affected because copper adsorbed to metal hydroxides cannot penetrate the cell membrane.

Amphipod growth was a sensitive indicator of copper toxicity, with ionic copper concentrations as low as 4 µg Cu/L causing a significant decrease in growth over 28 days. A

small but significant decrease in amphipod growth in station 14 water was observed, with no effect in station 9 water.

Although increases in copper content of flounder exposed to low concentrations of ionic copper were found, copper content of fish exposed to Macquarie Harbour waters for 14 days was the same as the controls. This supports the algal growth bioassays which suggested that much of the copper present in the Harbour was not bioavailable.

Ionic copper and Macquarie Harbour waters caused histopathological changes in juvenile flounder after 14-day exposures, including effects on the gills (epithelial necrosis, epithelial lifting and proliferation of chloride cells), liver (cloudy swelling) and kidneys (necrosis). However, apart from cloudy swelling in the liver, these effects were only observed at concentrations of ionic copper greater than that found in the Macquarie Harbour samples (>40 μg/L). It appears therefore that copper in the Macquarie Harbour waters was not the sole cause of these histopathological changes. Other metals or compounds in the Harbour waters may contribute to these effects.

Taking into account these results, and using the results of the risk analysis of literature values of copper toxicity in marine and estuarine waters, we estimate the maximum acceptable concentration of copper in Macquarie Harbour mid-depth waters to lie between 10 and 20 μg/L. This will require at least a four-fold reduction of dissolved copper from present levels.

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# 1 Introduction

The 100-year operation of the Mount Lyell Mining and Railway Company Limited's copper mine in Queenstown, Tasmania, has resulted in the deposition of over 100 million cubic metres of mine tailings, smelter slag and topsoil into the King River and Macquarie Harbour. Despite the cessation of tailings dumping, exposed tailings on the river banks and in the delta continually leach iron and copper, contributing substantially to the copper load in Macquarie Harbour. Although dissolved copper concentrations as high as 500 µg/L are found at some sites, much of this copper may be bound to organic and inorganic material and therefore not bioavailable.

The Mount Lyell Remediation, Research and Demonstration Program is undertaking a comprehensive study to assess the environmental impact of metal release from the mine and smelter, as part of the development of a remediation strategy. In particular, an assessment of the potential biological impact of elevated copper levels in Macquarie Harbour waters is required. Toxicity testing of Macquarie Harbour waters using appropriate species of algae, invertebrates and fish is one important component of this environmental assessment.

The objective of this project (project 9a) was to conduct toxicity tests to determine the concentration of copper which can be tolerated in Macquarie Harbour waters without causing detriment to aquatic life. Sensitive, sub-lethal tests using Australian temperate species of microalgae, invertebrates and fish were used to determine the toxicity of mid-salinity Macquarie Harbour waters. The mid-depth samples (20‰ salinity) represented the middle of the salt wedge boundary between the deeper, more dense seawater and the shallower, copper-contaminated river water. Mid-salinity waters were chosen for the toxicity tests because the marine species could tolerate these salinities and copper input from the overlying freshwater would be more soluble and hence potentially more bioavailable than in the deeper saltier waters.

The variation in total metal levels, metal speciation and salinity in Macquarie Harbour waters necessitated the careful selection of toxicity tests using sensitive Australian species. Ecotoxicological testing around the world has moved towards bioassays which measure sub-lethal, rather than lethal effects, and which use vulnerable early life stages of aquatic organisms from all levels of the food chain. Test species should be sensitive to metals, particularly copper, yet have a wide salinity tolerance. In addition, the species chosen should have well established test methodologies and preferably have been extensively used to provide a good database on expected no observed effect (NOEC) and lowest observed effect (LOEC) concentrations for various copper species.

One algal test species which fulfils all of these criteria is the estuarine diatom *Nitzschia closterium*, which is widely distributed in Australian coastal waters. This single-celled alga, isolated from temperate waters on the east coast of Australia, can tolerate salinities of 20‰. It is extremely sensitive to copper and can detect toxicity at copper concentrations as low as 5 µg/L. This chronic bioassay measures the decrease in cell division rate (growth rate) over 72 h in test water compared with controls. This bioassay has been used extensively throughout Australia and Papua New Guinea for determining the toxicity of metals, chemicals, natural waters and complex effluents for the mining, chemical and pulp/paper industries (Stauber 1995).

In addition to the algal growth bioassay with *Nitzschia*, an enzyme inhibition bioassay using the marine green alga *Dunaliella tertiolecta* was used the assess the toxicity of the Macquarie

Harbour waters. This bioassay measures the inhibition of the enzyme ß-D-galactosidase in the alga, after a 1-h exposure to toxicant (Peterson & Stauber 1996). In the assay, the algal enzyme cleaves the fluorogenic substrate 4-methylumbelliferyl-ß-D-galactoside (MU-gal), releasing the fluorescent compound 4-methylumbelliferone. The presence of toxicants in a water sample reduces this enzyme activity, causing a proportional reduction in fluorescence. This bioassay is particularly sensitive to copper, with a 1-h EC50 of 19 µg Cu/L (Stauber et al 1995).

The euryhaline amphipod *Allorchestes compressa* is a major component of decomposer food chains in southern Australian waters and is a primary food source for marine fish. This species has been used extensively since 1973 as a standard test species due to its sensitivity, ability to tolerate a wide range of salinities, short life history, and ease of maintenance in the laboratory (Ahsanullah 1976, 1982, Ahsanullah & Brand 1985, Ahsanullah & Florence 1984, Ahsanullah & Palmer 1980, Ahsanullah et al 1988). These studies have included acute and chronic toxicity tests, ranging from 4-day survival tests to 28-day growth tests and 30-week growth and reproduction tests with multiple generations. One advantage of the bioassay is that a large number of first instar juveniles can be obtained synchronously from the marsupia (brood pouches) of adult females using an anaesthetising procedure. The bioassay used in this study determines the effect of toxicants on the survival and growth of juveniles over 28 days in 10 L flow-through tanks.

Flounder (*Rhombosolea tapirina*) is a native fish with wide distribution in temperate Australian waters. Fourteen-day bioassays with juvenile flounder were carried out using survival, histopathology, copper accumulation and osmoregulation disturbance as endpoints.

In order to gain maximum benefit from ecotoxicological investigations, it is necessary to provide rational bases for assessing the environmental significance of the results. A preliminary risk assessment of the potential for toxic effects from copper in Macquarie Harbour was carried out by ANSTO. The aim of this risk assessment was to determine the degree of overlap between the distribution of measured copper concentrations in water samples from Macquarie Harbour and the distribution of concentrations of copper reported in the literature to have significant effects on biota in similar environments. By comparing these distributions, the probabilities of exceeding critical values of copper in the environment could be determined within set confidence limits.

A comprehensive literature review on the effects of copper on various marine and brackish water organisms is provided in this report. Information on acute, sub-lethal and chronic toxicity, together with data on the bioaccumulation of copper in Australian organisms, is included, with particular emphasis on the pivotal role played by the speciation of copper (ie its different physico-chemical forms) in the toxic effect.

# 2 Literature review

## 2.1 The chemistry and speciation of copper in natural waters

The total concentration of dissolved copper in surface, open ocean seawater is 0.03–0.15 µg/L, and in near-shore surface waters off Sydney, Australia, values of 0.09–0.3 µg Cu/L were found (Batley 1995). Similar values were found in Sydney estuarine waters (Batley & Gardner 1978), although higher copper concentrations up to 16 µg/L have been reported in other estuaries around the world (Mills & Quinn 1984). Carpenter et al (1991) reported 2–7 µg/L of dissolved copper in Macquarie Harbour, Tasmania. In unpolluted freshwater rivers and lakes, typical total dissolved copper concentrations were reported as 0.3–3 µg/L

(Florence 1977, Nriagu 1979). Carpenter et al (1991) found 0.3 μg Cu/L in the pristine Gordon River, Tasmania, but 31 μg/L of dissolved copper and 400 μg/L of particulate copper in the King River, which is affected by mine runoff. It is important to realise that total dissolved copper is, by definition, all physico-chemical forms of copper that will pass through a 0.45 μm membrane filter. This filterable copper will therefore include not only molecular forms of copper such as simple inorganic and organic complexes, but also 'pseudocolloids' of copper, where copper is adsorbed to colloidal particles of organic matter, manganese dioxide, and, especially, humic-coated iron oxide particles (Florence 1986b).

In open ocean surface seawater and in freshwaters with a high concentration of dissolved organic carbon (DOC), copper is largely (98–100%) complexed by humic matter and other natural adsorbents (especially colloidal humic-coated iron oxide particles) and complexing agents (Newell & Sanders 1986, Wangersky 1986, Sunda & Hanson 1987, Bruland et al 1991). When bound in this way, the metal is essentially non-toxic to aquatic organisms and will eventually be deposited in the bottom sediment of the water body. In other situations, however, significant concentrations of toxic copper species can be present. This applies to polluted rivers where the concentration of DOC is low compared with the concentration of copper, and to estuaries and near-shore seawater affected by sewage or industrial discharges. By performing a 'titration' of a water sample with a standard copper solution, and measuring the concentration of copper that needs to be added to the water sample before free copper ions appear, the copper complexing capacity of the water can be determined. The complexing capacity is a measure of the ability of substances in the water to bind copper in inert non-toxic complexes, so it is an important water quality parameter (Florence & Batley 1980, Florence 1986a, Morrison & Florence 1989). The endpoint of the titration is detected either by a bioassay (eg algal assay) or electrochemically (eg anodic stripping voltammetry (ASV) or copper ion selective electrode).

When inorganic forms of copper do exist in waters, the principal species in seawater are computed by equilibrium models to be the carbonato complex ($CuCO_3^0$, 82%), hydroxy complexes (6.5%), and free cupric ion (2.9%). In a typical freshwater, more than 90% of inorganic copper is computed to be present as $CuCO_3$ (Florence 1986a). All these inorganic forms of copper have a high toxicity to aquatic organisms.

A highly toxic form of copper which can be present in polluted waters is lipid-soluble (ie fat-soluble) copper. Most organic complexes of copper have low toxicity, but if the complex is lipid-soluble it can rapidly penetrate the cell membrane, allowing both copper and the ligand to enter the cell. Copper complexes of oxine and diethyldithiocarbamate are used as fungicides, and can find their way into river systems. Also, xanthates, used as mineral flotation agents, form strong, lipid-soluble complexes with copper (Florence 1982a, 1982b, 1983, Ahsanullah & Florence 1984, Florence & Stauber 1986, Stauber & Florence 1987, Florence et al 1992, Phinney & Bruland 1994).

All species of algae seem to produce exudates containing organic ligands capable of strongly binding copper (Fisher & Fabris 1982, Zhou et al 1989). This is not surprising, since copper is an essential nutrient for algae, and these organisms frequently produce organic compounds that are designed specifically to increase the bioavailability of certain essential metals. For example, siderophores are produced to solubilise iron and make it available for uptake by the algal cell. Fisher and Fabris (1982) and Zhou et al (1989) have suggested that the organic compounds which bind copper in marine waters originate from algal exudates.

### 2.1.1 Measurement of copper speciation

The determination of the speciation of copper in water samples is essential to understanding the toxicity of the water to aquatic animals, because different physico-chemical forms of copper that are present in water can have widely varying toxicities. In general, free hydrated cupric ion and readily dissociable copper complexes have the highest toxicity, whereas strong, inert complexes (with the exception of lipid-soluble complexes) have the lowest toxicity. It is not possible to measure the concentrations of all the individual copper complexes that are present in a water sample, because the act of measurement normally disturbs the equilibrium between the species in the sample. This, however, might not be a disadvantage, since the toxic action of copper on an organism is itself a dynamic process (Florence 1986a, 1992). The aim should be to determine the *toxic fraction* of copper in the water sample, ie the fraction of the total copper concentration that the organism recognises as toxic. The ideal speciation technique is one that would involve the use of an analytical probe which copper would react with at the same rate and with the same equilibrium constant as it does with a biological membrane (eg an algal cell) (Morrison & Florence 1988).

The limits of detection (3 times the standard deviation of the blank) of some sensitive analytical techniques for determining the *total* concentration of copper in seawater and freshwater samples are shown in table 2.1. The limit of quantitative determination (standard deviation ≈ 3–5%) is 5–10 times the detection limit.

Various techniques have been proposed for copper speciation in water samples, involving electrochemical methods (Florence 1982a, 1982b, 1983, 1986a, 1992, Florence & Mann 1987, Morrison et al 1989, Donat et al 1994, Paneli & Voulgraopoulos 1993) as well as ion exchange, chromatographic and solvent extraction methods (Florence & Batley 1977, 1980, Florence 1982a, Sunda & Hanson 1987, Zhang & Florence 1987, Morrison & Florence 1988). Of all these procedures, the ones that give the best correlation with algal assays are the 'double acidification' anodic stripping voltammetric procedure (Florence 1992) and the aluminium hydroxide ion exchange column method (Zhang & Florence 1987). The ion exchange column method is applicable to field processing of samples, and can measure separately the toxic fraction of copper and the concentration of lipid-soluble copper complexes in the sample. Rijstenbil and Poortvliet (1992) confirmed the usefulness of the aluminium hydroxide resin column for measuring the toxic fraction of copper in polluted estuarine water.

## 2.2 Ecotoxicology of copper to algae

### 2.2.1 Introduction

In both the oceans and freshwater systems, algae are the start of the food chain, or food web (Pimm et al 1991, Dakin 1987). Algae are high quality food, and are eaten not only by zooplankton and other tiny invertebrates, but also by larval stages of vertebrates (eg fish). Some huge marine animals such as the blue whale and the basking shark depend entirely on algae and the other constituents of plankton as a food source. Algae fix a major portion of the Earth's carbon, and generate, via photosynthesis, much of the oxygen in our atmosphere. Benthic algae help to aerate sediment via oxygen that they produce. Any pollutant that poisons algae and lowers their concentration in a natural water will therefore have important consequences for the health of the whole aquatic ecosystem.

**Table 2.1** Limits of detection for copper by some analytical techniques

| Technique [a] | Limit of detection (μg/L)[b] | |
|---|---|---|
| | Freshwater | Seawater |
| GFAAS | 0.5[c]<br>0.05[d] | -[c]<br>0.05[d] |
| ICP-AES | 1.0 | 2.0 |
| ICP-AES (AP) | 0.2 | 0.5 |
| ICP-MS | 0.005 | 10 |
| ASV | 0.2 | 0.2 |
| CSV | 0.1 | 0.1 |
| Spectrophotometry | 2.0 | 2.0 |

a GFAAS: Graphite furnace atomic absorption spectroscopy; ICP-AES: Inductively-coupled plasma atomic emission spectroscopy; AP: Axial plasma; ICP-MS: Inductively-coupled plasma mass spectrometry; ASV: Anodic stripping voltammetry; CSV: Cathodic stripping voltammetry; Spectrophotometry: bathocuproine method

b Detection limit: 3 x standard deviation of blank

c Direct determination

d After pre-concentration

Some forms of algae are exquisitely sensitive to toxicants (Florence & Stauber 1991), especially heavy metals, and their viability can be used as an indicator of environmental change. Diatoms, in particular, are a useful and convenient organism to detect even low levels of environmental stress because they are so abundant and widely distributed (Dixit et al 1992). On the other hand, an explosion in the algal population in a water system (an 'algal bloom') has serious environmental consequences. The mass of dead and decaying algae reduces the concentration of dissolved oxygen in the water as well as producing substances which are toxic to fish and other organisms. The 'red tides' that appear periodically off beaches in the north of Australia and cause large fish kills are caused by species of dinoflagellates which, as they die, release chemicals that are highly toxic. Treatment with copper sulphate is the most common technique used to control algal blooms in drinking water reservoirs. However, some species of algae (cyanobacteria), when poisoned by copper, release neurotoxins and hepatotoxins which have caused extensive poisoning of livestock and wildlife (Kenefick et al 1993).

Based on free metal ion activity (not total dissolved metal concentration), the order of toxicity of metals to algae is usually found to be (Canterford & Canterford 1980):

$Hg^{2+} > Ag^{+} > Cu^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+} > Tl^{+}$

However, because mercury, silver and copper are strongly bound by (or adsorbed to) organic matter in natural waters, and lead is largely hydrolysed, cadmium, which is only weakly complexed by organic ligands and less hydrolysed, is often found to be the most toxic metal on the basis of total dissolved metal. This is one reason why the speciation analysis of metals in water samples is so important; strongly-bound metals usually exhibit low toxicity, so the potential toxicity of dissolved metals cannot be assessed if only total metal concentrations are available (Morrison & Florence 1989).

In water systems affected by heavy metal pollution, copper is commonly the metal of most concern. For this reason, many of the studies of metal toxicity to algae have involved copper. Regulatory bodies have published maximum allowable concentrations of copper for both marine and freshwater, and some of these criteria are shown in table 2.2.

**Table 2.2** Some regulatory body criteria for copper (protection of sensitive aquatic organisms)

| Regulatory body[a] | Limiting copper concentration (µg/L) | | | |
|---|---|---|---|---|
| | Acute toxicity | | Chronic toxicity | |
| | Freshwater | Seawater | Freshwater | Seawater |
| ANZECC | | | 2–5[b] | 5 |
| USEPA | 9.2[b] | 2.9 | 6.5[b] | 2.9 |
| UKWQS | | | 1–28[b] | 5 |

a ANZECC: Australian and New Zealand Environment and Conservation Council (1992); USEPA: United States Environment Protection Agency (1986); UKWQS: United Kingdom Water Quality Standards (Foundation for Water Research 1989)

b Depends on water hardness

### 2.2.2 Bioaccumulation of copper by algae

All species of algae show a marked ability to accumulate copper, and the amount of copper adsorbed by algal cells depends on the concentration of copper in solution, and ranges from 1 µg Cu/g dry algal mass for algae in the pristine area of the Great Barrier Reef (Denton & Burdon-Jones 1986a), to 300 µg Cu/g in polluted waters. Some results for copper accumulation by marine algae (from Denton & Burdon-Jones 1986a) are shown in table 2.3.

**Table 2.3** Bioaccumulation of copper by marine algae[a]

| Algal species | Sampling location | Copper (µg/g) dry wt |
|---|---|---|
| *Dictyota volubilis* | Townsville | 19 |
| *Lobophora* sp | Townsville Harbour | 24 |
| *Padina tenuis* | Townsville Harbour | 8 |
| *Caulerpa racemosa* | Townsville | 3 |
| *Dictyosphaeria versluysii* | Townsville | 3 |
| *Dictyosphaeria versluysii* | Great Barrier Reef | 2 |
| *Dictyota bartayressii* | Penang, Malaysia | 50 |
| *Sargassum pallidum* | Pacific Coastal Waters | 3 |
| *Caulerpa racemosa* | Penang, Malaysia | 7 |
| *Hypnea musciformis* | Goa, India | 7 |

a from Denton and Burdon-Jones (1986a)

When copper ions are added to suspensions of algae, the metal is rapidly absorbed by the cell surface (Hawkins & Griffiths 1982). Florence et al (1983) found that 20–45% of added copper sulphate (5–50 µg Cu/L) adsorbed within one hour to a suspension (2–4 x $10^7$ cells/L) of the marine diatom *Nitzschia closterium*, in seawater. With this diatom, copper binds to the cell with an equilibrium constant in the range log $\beta_1 < 13 > 10$, and the bonding is by protein amino and carboxylic acid groups (Florence et al 1983, Florence & Stauber 1986). These authors concluded that thiol groups must not be involved because, if they were, the equilibrium constant would be orders of magnitude higher. Gonzalezdavila et al (1995), using the marine green flagellate *Dunaliella tertiolecta* found that copper was bound to the cell wall by two major functional groups, one with a high affinity for copper, and another with low affinity. The high-affinity binding constant was of the same order of magnitude as the copper complexing ability of the exudate excreted by the alga. Florence et al (1983) also found with *Nitzschia closterium* that whereas copper bound to the algal cells with low concentrations of added copper (<1 x $10^{-3}$ ng Cu/cell) could not be removed electrochemically, a significant fraction of the cell-bound copper was electrochemically-available when higher amounts were bound.

Several authors (eg Hardstedt-Romeo & Gnassia-Barelli 1980) found that algal exudates bind copper and reduce the amount of metal bioaccumulated.

### 2.2.3 Mechanisms of toxicity of copper to algae

The cellular responses of aquatic organisms to pollutants, including copper, have been reviewed by Luoma (1983) and Moore (1985). Heavy metals such as copper are normally transported into an algal cell by the process of facilitated diffusion or host-mediated transport, where a carrier protein on the surface of the cell membrane removes copper from its hydrophilic complex (CuL) in solution, binds it in a more hydrophobic complex that can traverse the hydrophobic region of the membrane, then releases it inside the cell (the cytosol) where it is probably bound as Cu(I) by thiol groups. The carrier protein then returns to the cell surface to collect another copper ion.

Solution: CuL ------- protein-membrane : Cell

CuL + protein —> Cu-protein + L (solution)

Cu-protein —> Cu-Cell + protein-membrane

Note that in the process of facilitated diffusion, copper is retained in the cell but the ligand that was bound to it in solution remains in solution. Stauber and Florence (1986, 1987) found that the most likely toxic mechanism of ionic copper is lowering of the ratio of reduced to oxidised glutathione (GSH/GSSG) in the cell (Rijstenbil et al 1994a). Lowering this ratio decreased cell division rate in *Nitzschia closterium* but did not affect photosynthesis (Florence & Stauber 1986, 1987). Fisher et al (1981) also observed that copper had no effect on photosynthesis in *Asterionella japonica*, but depressed cell division, leading to 'giant' cells. In the marine diatom *Asterionella glacialis* and the freshwater green alga *Chlorella pyrenoidosa*, however, ionic copper depressed both photosynthesis and cell division rate (Stauber & Florence 1987). Intracellular copper can also affect the production of ATP and inactivate several enzymes (Stauber & Florence 1987, Cid et al 1995). In *Nitzschia closterium*, at least, the site of copper action is not the chloroplast or the mitochondria, but is concentrated in the cytoplasm (Stauber & Florence 1987).

This convoluted facilitated diffusion process is not required if the copper complex in solution is lipid soluble. Lipid soluble copper complexes can diffuse directly through the hydrophobic cell membrane without having to first dissociate. Not only is this process much faster than facilitated diffusion (so more copper can accumulate in the cell), but the ligand is also transported into the cell where it might cause its own toxic effect. The toxic effect of lipid-soluble copper complexes might well occur in the cell membrane rather than in the cytosol. Florence et al (1983), Florence and Stauber (1986), and Stauber and Florence (1987) found that lipid-soluble copper complexes such as copper oxinate were bioaccumulated from solution by *Nitzschia closterium* to the extent of about 80%, compared with 20% absorption of copper from copper sulphate solution. Phinney and Bruland (1994) confirmed these results and found that the coastal diatom *Thalassiosira weissflogii* accumulated copper from its oxinate complex at ten times the rate it did from its complex with the water-soluble sulphonated derivative of oxine.

Toxicity of copper to *Nitzschia closterium* was ameliorated by even very low concentrations of iron (III), manganese (III), cobalt (III) and other trivalent metals in the growth medium (Stauber & Florence 1985a, 1985b, 1987). The algal cells produce superoxide radical and hydrogen peroxide which oxidise Mn(II) and Co(II) respectively at the cell surface. The trivalent metals form a layer of metal hydroxide around the cell, adsorb copper ions and reduce their penetration into the cell. As little as 2.5 µg/L of cobalt, 5 µg/L of manganese, 50 µg/L of iron or 100 µg/L of aluminium in the growth medium reduces the toxicity of copper to *Nitzschia closterium* by a factor of ten. The trivalent metals did not afford any protection against copper to the freshwater green alga, *Chlorella pyrenoidosa*, presumably because the pH of the growth medium (pH 7.0) was too low to promote sufficient hydrolysis of the metals (Stauber & Florence 1987).

### 2.2.4 Toxicity of copper to marine algae

The toxicity of copper to different species of marine algae varies greatly (Fisher 1977, Hodson et al 1979); a collection of some published EC50 values (ie the effective concentration (EC) of copper which reduces the rate of cell division by 50%) is given in tables 2.4–2.6. In the case of laboratory assays, the EC of copper is simply the total concentration of added copper. It is important to realise, however, that most of the laboratory algal assays that produced these EC50 values were carried out in full culture media. As shown by Lumsden and Florence (1983) and Stauber and Florence (1989), this procedure can seriously underestimate the toxicity of copper, because culture media contain high concentrations of substances such as phosphate, silicate, EDTA, manganese, cobalt and iron (Steeman Nielsen & Wium-Andersen 1970, Morel et al 1978, Florence et al 1983, Stauber & Florence 1985a) which complex or adsorb copper and reduce its bioaccumulation and toxicity. Natural waters contain very much lower concentrations of these binding agents.

In all the algal assays used in the research by Florence, Stauber, and co-workers, the growth medium for marine algae was seawater with no additions whatsoever, and the inoculum of algae was washed three times with seawater by centrifugation before use. In the case of the marine diatom, *Nitzschia closterium*, Stauber and Florence (1989) found an EC50 value for copper of 10 µg/L in unenriched seawater, but a value of 200 µg Cu/L when the assay was carried out in medium f. The exact procedure used for algal assays by Stauber and Florence is given in all of their publications.

**Table 2.4** Toxicity of copper to marine phytoplankton (1)

| Algal species | Medium [a] | Reference | EC50 (µg Cu/L)[b] |
|---|---|---|---|
| *Nitzschia closterium* | sw, 32 ‰ | Stauber & Florence (1989) | 10 |
| | f | Stauber & Florence (1989) | 200 |
| *Phaeodactylum tricornutum* | sw, 35 ‰ | Cid et al (1995) | 100 |
| | S88 | Bentley-Mowatt & Reid (1977) | 5000 |
| *Skeletonema costatum* | Aquil | Metaxas & Lewis (1991a) | 30 |
| *Nitzschia thermalis* | Aquil | Metaxas & Lewis (1991a) | 35 |
| *Dunaliella tertiolecta* | Droop | Overnell (1975) | 600[c] |
| *Tetraselmis* sp | S88 | Bentley-Mowatt & Reid (1977) | 6000 |
| *Dunaliella primolecta* | S88 | Bentley-Mowatt & Reid (1977) | 6000 |
| *Hymenomonas elongata* | S88 | Bentley-Mowatt & Reid (1977) | 6000 |
| *Asterionella japonica* | f/2 | Fisher et al (1981) | 7 |
| *Ditylum brightwellii* | f/2, 14 ‰ | Rijstenbil & Wijnholds (1991) | 8 |
| *Nitzschia palea* | sw + nutrients | Steeman Nielsen and Wium-Anderson (1970) | 10 |

a sw = seawater

b EC50 – copper concentration for 50% depression of cell division rate

c 50% depression of photosynthesis

**Table 2.5** Toxicity of copper to marine phytoplankton (2)[a]

| Algal species | EC50 [b] (µg Cu/L) |
|---|---|
| *Bellerochea polymorphia* | 6400 (9.6)[c] |
| *Skeletonema costatum* | 6200 (9.7) |
| *Thalassiosira guillardii* | 3200 (10.3) |
| *Thalossiosira pseudonana* | 4200 (10.2) |
| *Monallantus salina* | 58000 (8.7) |
| *Nannochloris atomus* | 46000 (8.9) |
| *Pavlova lutheri* | 5100 (10.0) |

a From Gavis et al (1981); Algal assays carried out in modified f/2 medium (containing EDTA).

b EC50 – 50% depression of cell division rate.

c Number in brackets is pCu, the negative logarithm of free cupric ion activity (calculated).

**Table 2.6** Toxicity of copper to marine phytoplankton (3)[a]

| Algal species | EC50 [b] (µg Cu/L) |
|---|---|
| *Asterionella glacialis* | 190 (10.5)[c] |
| *Ditylum brightwellii* | 450 (10.0) |
| *Skeletonema costatum* | 640 (9.7) |
| *Thalassiosira pseudonana* | 450 (10.0) |
| *Hymenomonas carterae* | 320 (10.2) |
| *Thoracosphaera heimii* | 45 (11.1) |
| *Synechococcus* sp (WH 7808) | 90 (10.7) |

a From Brand et al 1986. Growth medium was a modified f/2 (containing NTA).

b EC50 copper concentration for 50% depression of cell division rate.

c Number in brackets is pCu, the negative logarithm of free cupric ion activity (calculated).

It has been shown repeatedly, under laboratory conditions using well-defined growth media containing EDTA where the free cupric ion activity can be computed fairly accurately, that the toxicity of copper to phytoplankton is related to copper activity (pCu) rather than to total copper concentration (Sunda & Guillard 1976, Petersen 1982, Lage et al 1994). Whether this is always true in field water conditions is debatable. In the laboratory experiments, pCu is calculated from thermodynamic equilibrium constants, ie the assumption is made that the test solution is at equilibrium. Kinetic factors are not considered. However, natural waters contain a wide range of ligands and metal complexes, and dissolved copper can be bound to humic, fulvic, tannic, and alginic acids, as well as to various inorganic and organic colloidal particles. All of these copper complexes dissociate at different rates, some quite slowly, so that equilibrium between an algal cell and a molecule of a copper complex might not be attained in the contact time that occurs in a natural water system.

The toxicity of copper to algae is ameliorated by exudates produced by the algal cells (McKnight & Morel 1979, Van den Berg et al 1979, Fisher & Fabris 1982, Brown et al 1988, Zhou et al 1989, Rijstenbil & Wijnholds 1991, Gerringa et al 1995). The amount of exudate produced by the cells increases with increasing copper concentration. The conditional stability constant of copper with these exudates in seawater has been measured as being in the range log K' = 8.5–10.0. Gerringa et al (1995), studying the marine diatom *Ditylum brightwellii*, found that free copper concentration in solution was buffered at about 0.01 µg/L by the exudate up to a maximum dissolved copper concentration of 7 µg/L. At higher copper concentrations, toxicity increased rapidly. However, both Brown et al (1988) and Gerringa et al (1995) concluded that adsorption of copper by mucilage on the external surface of the cell was a more important detoxification mechanism than complexing of copper by exudate. The algal exudates also seem capable of removing some copper which is adsorbed to suspended particulate matter (Florence 1986b).

Another factor which makes the transfer of laboratory algal assay toxicity results to natural waters difficult is that the bioassays are normally carried out using a single algal species, whereas in natural waters a great many species of algae co-exist. Under these natural circumstances, the different algae compete with one another for the available nutrients and attempt to dominate by producing exudates that are toxic to other algal species but not to themselves. De Jong and Admiraal (1984) found that in mixtures of the two marine diatoms, *Amphiprora paludosa* and *Nitzschia closterium*, the *Amphiprora paludosa* cells produced a substance which inhibited the growth of *Nitzschia closterium*. Likewise, Metaxas and Lewis (1991a) found that the marine diatom *Skeletonema costatum* would not grow in the presence of another diatom, *Nitzschia thermalis*, which produced an inhibitory exudate. Goldman and Stanley (1974) found that, in general, marine diatoms will dominate other marine phytoplankton such as the centric diatom *Skeletonema costatum*. In the English Trent River system, two species of green algae and one diatom were the first to colonise tributaries downstream of a copper processing plant discharging copper to the stream. No micro-flora were observed within 6.4 km of the plant outfall (Hodson et al 1979).

There is a considerable amount of information on the effects of commercial copper-based algicides (Fitzgerald 1975). These commercial products are usually more effective, on a copper basis, than simple copper sulphate because they contain copper complexing agents such as triethanolamine which help to keep copper in solution by preventing the formation and precipitation of hydrolysed copper compounds.

### 2.2.5 Toxicity of copper to freshwater algae

As for marine algae, freshwater phytoplankton species show a considerable variation in their sensitivity towards copper, although marine algae tend to be more sensitive towards copper than freshwater algae (Nriagu 1979). Both cell division rate and photosynthesis are inhibited. Hutchinson (1973) found that copper, at concentrations higher than 50 µg/L, was toxic to *Chlorella vulgaris, Scenedesmus acuminata, Chlamydomonas eugametas* and *Haematococcus capensis*. In the case of *H. capensis*, however, copper actually *stimulated* growth up to a concentration of 100 µg/L, then was inhibitory at higher copper concentrations (hormesis effect). Schafer et al (1993) found an EC50 of about 80 µg/L copper for the green alga *Chlamydomonas reinhardii*, while Stauber and Florence (1989) reported an EC50 of 15 µg/L for the green alga *Chlorella pyrenoidosa* (Chick). The composition of the growth medium, as in the case of marine algae, has a profound effect on the toxicity of copper, since chelators such as EDTA will chelate copper and decrease its toxicity (Steeman Nielsen & Wium-Andersen 1970). Stauber and Florence (1989) found EC50 values (µg Cu/L) of 16, 24 and >200 in synthetic soft water, EPA medium, and MBL medium (no EDTA) respectively. Monahan (1976), using a growth medium containing EDTA, found that 500 µg/L of copper was required to cause toxicity to *C. pyrenoidosa*.

Natural and synthetic chelating agents affect the toxicity of copper to freshwater algae. Morrison and Florence (1988) found that NTA and fulvic acid (but not an iron-humic colloid) decreased the toxicity of copper to *Chlorella pyrenoidosa*, while Garvey et al (1991) reported that humic acid, but not fulvic acid, lowered the toxicity of copper to the green alga *Chlamydomonas reinhardii*. Soil-derived humic acid was found to be more effective than aqueous humic acid (Shanmukhappa & Neenakantan 1990). In contrast to these results, Tubbing et al (1994) found that the copper-EDTA complex was able to inhibit photosynthesis of *Selenastrum capricornutum*. These authors concluded that *complexed* copper is available to algal cells, and that the fraction of copper in solution that is absorbed by a column of Chelex-100 resin is similar to the bioavailable fraction. Morrison and Florence (1988) found that the aluminium hydroxide resin column technique (Zhang & Florence 1987) provided measurements of reactive copper which correlated well with bioassays using *Chlorella pyrenoidosa*. In general, copper is more toxic to phytoplankton in soft than in hard waters (Nriagu 1979).

Some other factors which affect the toxicity of copper to freshwater algae are seasonal variability, synergistic interaction of metals, laboratory selection, and the size of the algal biomass. Winner and Owen (1991), using enclosures in a natural freshwater pond, found that all algal divisions, with the exception of the Cryptophyta, showed seasonal sensitivity to copper, with highest sensitivity being in the spring. Wong and Chang (1991) showed that, in a modified complete medium (containing EDTA), chromium (VI), ie chromate, and nickel strongly increased the toxicity of copper to *Chlorella pyrenoidosa* 251. In the absence of these other two metals, the EC50 for copper was about 270 µg Cu/L, and 100 µg Cu/L caused only 7% depression of cell division rate. The addition of 100 µg/L of chromium (VI), which alone had no effect on growth, caused complete suppression of growth in the presence of 100 µg Cu/L. Likewise, 100 µg/L of nickel, non-inhibitory alone, completely suppressed growth when 100 µg/L of copper was added.

Twiss et al (1993), using the microalga, *Scenedesmus acutus* f *alternans*, showed that tolerance towards copper could be substantially increased by repeated culturing of the alga in media containing sub-lethal concentrations of copper. The copper-tolerant isolate absorbed nearly twice as much copper as the parent strain.

Swartzman et al (1990) found that the toxicity of copper to ten phytoplankton and five zooplankton species in a controlled microcosm was dependent on the total algal biomass. When the biomass was high, concentrations of copper were tolerated which devastated algal populations of low abundance. This increased tolerance towards copper was ascribed to decreased bioavailability of copper as a result of adsorption and chelation by exudates, and to an increase in pH, which lowered the concentration of free cupric ion (Nriagu 1979).

Oliveira (1985) studied the effect of a mine effluent rich in copper on the structure of the phytoplankton community in a chain of low alkalinity and low hardness reservoirs in Portugal. Compared with an uncontaminated reservoir (4 µg Cu/L), a shift in the dominant species occurred under the influence of the effluent. The control reservoir was characterised by Chlorocococales and diatoms, whereas the effluent-affected reservoirs (5–25 µg Cu/L) had dominant phytoplankton populations of the desmid type. Wong et al (1995) proposed techniques for identifying the sources of toxicity in wastewaters using algal bioassays.

## 2.3 Ecotoxicology of copper to corals and sea anemones

Harland and Ngarno (1990) reported that exposure of the sea anemone *Anemonia viridis* to copper caused an immediate retraction of tentacles and copious production of mucus in both normal and aposybiotic anemones. After 24 h, tentacles were partially extended and mucus secretion decreased. In symbiotic anemones, there was progressive visible bleaching and loss of zooxanthellae over the 5-day experimental period. The bleaching effect was more extreme in anemones exposed to 200 µg Cu/L than to 50 µg Cu/L.

Harland and Ngarno (1990) also investigated the uptake of copper by the sea anemone *Anemonia viridis* and the role of zooxanthellae (symbiotic algae) in metal regulation. When exposed to 50 and 200 µg Cu/L in seawater, anemones did not take up the metal in proportion to external concentrations. Results suggested that *A. viridis* regulated copper by expelling zooxanthellae which were shown to accumulate copper. The use of non-zooxanthellae (aposymbiotic) anemones in similar metal uptake experiments indicated that other mechanisms may also be involved in metal regulation. Mucus was produced by *A. viridis* when the anemone was exposed to copper and this mucus may be involved in the regulation process.

Esquivel (1986) carried out a copper bioassay on planulae of the coral *Pocillopora damicornis* and reported that when the planulae were exposed to between 10 and 100 µg Cu/L, zooxanthellae were expelled and mucus produced. Howard et al (1986) exposed the coral *Montipora verrucosa* to a range of copper concentrations and found that between 10 and 500 µg Cu/L, the number of expelled zooxanthellae increased in proportion to copper concentration (Esquivel 1986, Howard et al 1986 cited by Harland & Ngarno 1990).

Heyward (1988) investigated the effects of copper sulphate (and zinc) on the fertilisation success in three species of corals in Japan. The impacts of copper and zinc sulphates on fertilisation success were reported. Gametes were collected from colonies of *Gonistrea aspera*, *Favites chinensis* and *Platygyra ryukyuensis* and used to create cross-fertilisation trials in replicate pairs of 1 L plastic bottles. All treatments were sampled 4.5 h after insemination and percentage fertilisation determined. Initial experiments with high concentrations of copper sulphate demonstrated complete inhibition of fertilisation for all three species in solutions containing greater than or equal to 500 µg/L copper (nominal). Controls of *F. chinensis* achieved a mean of 89.5% fertilisation, copper at 100 µg/L resulted in 45.7% fertilisation, while treatment with copper at concentrations less than or equal to 0.01 mg/L showed no significant deviation from the controls. However, Denton and Burdon-Jones (1986c) have analysed various octocorallian and seleractinian corals from within the Great Barrier Reef province for copper and other metals. Of the two coral groups, the octocorals accumulated significantly greater amounts of all detectable metals.

## 2.4 Ecotoxicology of copper to crustaceans

### 2.4.1 Toxicity

Numerous studies have been carried out on the toxic effects of copper on various Australian marine crustaceans (table 2.7). These include three species of copepods (Arnott & Ahsanullah 1979), larvae of the crab *Paragrapsus quadridentatus* (Ahsanullah & Arnott 1978), the burrowing shrimp *Callianassa australiensis* (Ahsanullah et al 1981a, b), the marine amphipod *Allorchestes compressa* (Ahsanullah & Florence 1984), the leader prawn *Penaeus monodon* and the banana prawn *P. mergiensis* (Denton & Burdon-Jones 1982, Ahsanullah & Ying 1995) and larvae and juveniles of the sand crab *Portunus pelagicus* (Mortimer & Miller 1994).

Arnott and Ahsanullah (1979) examined the effect of copper on three species of copepods *Scutellidium* sp, *Paracalanus parvus* and *Acartia simplex* in a 24 h exposure. The LC50 (the median lethal concentration) values for the three species ranged from 180 to 200 µg Cu/L. Similar toxicity of copper to larvae of the crab *P. quadridentatus* was observed (96-h LC50 of 170 µg Cu/L) despite different exposure times. Mortimer and Miller (1994) also reported on the susceptibility of larvae and juvenile instars of the sand crab *Portunus pelagicus* to various metals including copper. The 24-h LC50 to zoea 3 (a moulting stage) was 110 µg/L. Larval moulting was significantly inhibited by an exposure of only 30 µg Cu/L.

**Table 2.7** The median lethal concentrations (mg/L) of copper for various species (from Arnott & Ahsanullah 1979, Ahsanullah & Arnott 1978, Ahsanullah & Florence 1984, Ahsanullah et al 1981a)

| Species | Duration of experiment (h) | LC50 |
|---|---|---|
| Copepod | | |
| *Scutellidium* sp | 24 | 0.18 |
| *Paracalanus parvus* | 24 | 0.19 |
| *Acartia simplex* | 24 | 0.20 |
| Crab larvae | | |
| *Paragrapsus quadridentatus* | 96 | 0.17 |
| Amphipod | | |
| *Allorchestes compressa* | | |
| Adult | 96 | 0.50 |
| Juvenile | 96 | 0.11 |
| Burrowing Shrimp | | |
| *Callianassa australiensis* | 96 | 1.03 |
| | 168 | 0.34 |
| | 240 | 0.22 |
| | 336 | 0.19 |

Acute toxicities of eleven metals, including copper, to early life-history stages of the yellow crab *Cancer anthonyi* were determined by Macdonald et al (1988). They reported that copper completely inhibited hatching of all embryos at ≥100 µg Cu/L and significantly reduced hatching at 10 µg/L, ie 100 times lower than concentrations causing mortality.

Bjerregaard and Vislie (1986) investigated the effects of copper on ion and osmoregulation in the shore crab *Carcinus maenas* and reported that 1 mg Cu/L resulted in 50% mortality in 11 to 22 days and the highest sensitivity was observed around the moulting period. They further reported that 0.5, 1.0 and 10 mg Cu/L altered regulation of osmolality and $Na^+$, $K^+$ and $Cl^-$ concentrations, with no effect at 0.25 mg Cu/L.

The Australian marine amphipod *Allorchestes compressa* has been used in a variety of ecotoxicological investigations, including acute toxicity tests (Ahsanullah 1976, 1982, Ahsanullah & Palmer 1980, Ahsanullah & Florence 1984, Ahsanullah et al 1988) and sub-lethal studies (Ahsanullah & Brand 1985, Ahsanullah & Williams 1986, 1991). For copper sulphate, 96-h LC50 values of 110 and 500 µg Cu/L were determined for the juveniles and the adult amphipods respectively, with juveniles being 4 to 5 times more sensitive to copper than adults. Organocopper complexes were tested on adults only. The three water-soluble ligands nitrilotriacetic acid, 8-hydroxyquinoline-5-sulphonic acid and tannic acid ameliorated copper toxicity by decreasing the concentration of free ionic copper, while lipid-soluble ligands such as oxine and potassium ethylxanthogenate increased copper toxicity, presumably as the result of the complexes diffusing through the cell membrane and participating in injurious reactions. The copper complex with 2, 9-dimethyl-1,10-phenanthroline was the most toxic complex tested. It was suggested that the presence of these ligands in the receiving water should be taken into consideration when establishing water-quality criteria.

Ahsanullah et al (1981a) investigated the acute toxicity of copper (together with zinc and cadmium) to the burrowing shrimp *Callianassa australiensis* in semi-static tests. Each test lasted up to 14 days and the LC50 values were calculated for 4, 7, 10 and 14 day exposures. The toxicity of copper increased with exposure time, thus the 4-day LC50 value of 1.03 mg Cu/L was considerably higher than the 14-day LC50 of 0.19 mg Cu/L. Individual, paired and triad combined toxic effects of zinc, cadmium and copper were determined on *C. australiensis* (Negilski et al 1981) and *A. compressa* (Ahsanullah et al 1988). *Callianassa australiensis* (Dana) that survived 14 d acute lethality studies (Ahsanullah et al 1981a) were analysed to determine the concentrations of zinc, cadmium and copper in the whole shrimp and in various parts of the body. Using regression analysis, the influence of each metal upon the uptake of the others was studied. In a mixture of zinc and copper, the uptake of zinc was enhanced and that of copper was inhibited. In a mixture of cadmium and copper, the uptake of copper was inhibited by the presence of cadmium, but cadmium uptake was unaffected in the presence of copper. In a mixture of all three metals, similar effects were observed except that zinc and copper, occurring together, appeared to have no effect upon cadmium uptake. The variation in concentration factors was described and the need for further research on the effects of combinations of metals on various organisms was highlighted (Ahsanullah et al 1981b).

Virtue et al (1992) examined several aspects of copper toxicity to the Antarctic krill, *Euphansia superba*. Baseline copper concentrations in krill were found to be in the range 55.2–82.6 µg/g, dry weight. This species was able to regulate copper to a constant level beyond which copper became lethal at estimated ambient bioavailable copper ion concentrations of 0.9 µg/L at an LT50 (survival time for 50% of the exposed population) of 3.25 days. Krill died when the total body concentration reached approximately 250–300 µg/g dry weight. The same authors exposed krill to copper with and without the chelating agent NTA and reported that the LT50 for animals at 63 µg Cu/L was 2.45 days, increasing to 19 days with the addition of NTA.

Denton and Burdon-Jones (1982) investigated the influence of temperature and salinity on the acute toxicity of various metals including copper to juveniles of the banana prawn, *Penaeus merguiensis* (table 2.8). They concluded that the toxicity of all metals increased with increasing temperature. This was most noticeable in the high salinity treatment particularly for copper (and zinc).

The toxicity of dissolved copper at 4 different salinities (table 2.9) to the juvenile banana prawn *Penaeus merguiensis* has also recently been investigated by Ahsanullah (in prep). The 96-h LC50 values ranged from 280 to 720 µg/L at 15 to 30‰ salinity, decreasing with decreasing salinity. Ahsanullah also studied the toxicity of particulate copper. The exposure of *P. merguiensis* juveniles for 9 weeks to particulate copper concentrations ranging from 200 to 10 000 µg/g at 20‰ and 30‰ salinities had no significant effect on the mean size, average weight, percentage survival or standardised biomass (% survival x average weight) of the prawns.

**Table 2.8** The effects of temperature and salinity upon copper toxicity to juvenile *Penaeus merguiensis* (from Denton & Burdon-Jones 1982)

| Temp (°C):Sal(°/₀₀) | 96-h LC50 (µg/L) | 95% Confidence limits |
|---|---|---|
| 35 : 36 | 350 | 0.18-0.67 |
| 35 : 20 | 210 | 0.14-0.32 |
| 30 : 36 | 900 | 0.50-1.62 |
| 30 : 20 | 530 | 0.25-1.10 |

**Table 2.9** The effect of salinity upon copper toxicity to juveniles of the banana prawn *Penaeus merguiensis* at 20°C

| Salinity | 96-h LC50 (µg/L) | |
|---|---|---|
| ($^{o}/_{oo}$) | LC50 | 95% confidence limits |
| 15 | 280 | 0.08–0.95 |
| 20 | 380 | 0.10–1.4 |
| 25 | 520 | 0.13–2.1 |
| 30 | 720 | 0.15–3.3 |

Ahsanullah and Ying (1995) also assessed the effects of dissolved copper on *Penaeus merguiensis* and *P. monodon*. The 96-h LC50 and its 95% confidence limits for *P. merguiensis* were 380 and 210–680 µg Cu/L respectively. For *P. monodon*, mortality was less than 50% in copper concentrations between 0.5 and 2.5 mg/L. After 2 weeks exposure, the percentage survival of animals in the control tanks for *P. monodon* and *P. merguiensis* were 100% and 80% respectively, while the survival rate for both species in the highest Cu concentration decreased. The survival of *P. merguiensis* was less than that of *P. monodon* in all test solutions. Dissolved copper significantly affected the growth of *P. merguiensis* ($P<0.05$) but not of *P. monodon*. There was no significant effect of copper on the growth of *P. merguiensis* in tanks containing 50 µg Cu/L, while those in 100, 150 and 200 µg Cu/L tanks grew significantly slower than those in control tanks ($P<0.05$). *P. monodon* grew in all copper concentrations at the same rate as in control solutions.

### 2.4.2 Bioaccumulation

The distribution of copper and other heavy metals in two species of wild prawns, *Macrobrachium rosenbergi* and *M. novaehollandiae,* from the brackish part of the Adelaide River, Northern Territory, was investigated by Peerzada et al (1992). The authors divided the prawns into size groups of various carapace length (CL) for *M. novaehollandiae* and a single 22.52 mm CL size group for *M. rosenbergi*. They reported that the highest concentration of copper was in the hepatopancreas followed by ovaries, gills and muscles (table 2.10).

Darmono and Denton (1990) measured the heavy metal concentrations in juveniles of the banana prawn *Penaeus merguiensis* and the leader prawn *P. monodon* from the Townsville region, Queensland. They collected juveniles of *P. merguiensis* from the estuaries of Three Mile Creek (TMC) and the mouth of the Bohle River Creek (BRC), near a nickel refinery. *P. monodon* was collected from an aquaculture farm. Muscles of both species of prawns contained the lowest levels of copper and the hepatopancreas contained the highest levels. In the case of *P. monodon*, gill tissues contained the highest concentration of copper, followed by hepatopancreas and muscles. The authors further indicated that although *P. merguiensis* from both locations were of a similar size range, they were collected at different seasons (dry and wet season) and therefore temporal differences may have obscured or enhanced the relatively small variation between sites. Nevertheless, the significantly higher levels of copper in the hepatopancreas and in the gills from the prawns from Three Mile Creek may well be attributed to their close proximity to urban development, as well as to boating and other recreational activities (table 2.11).

**Table 2.10** Mean concentrations of copper (µg/g wet wt) in *Macrobrachium rosenbergi* and *Macrobrachium novaehollandiae* from the Adelaide River (from Peerzada et al 1992)

| Tissue | Carapace length (mm) | Copper (µg/g wet wt) |
|---|---|---|
| Muscles | | |
| *Macrobachium novaehollandiae* | 15–19 | 7.2 |
| *Macrobachium novaehollandiae* | 19–22 | 7.2 |
| *Macrobrachium rosenbergi* | 22–52 | 8.3 |
| Hepatopancreas | | |
| *Macrobrachium novaehollandiae* | 15–19 | 360 |
| *Macrobrachium novaehollandiae* | 19–22 | 330 |
| *Macrobrachium rosenbergi* | 22–52 | 310 |
| Ovaries | | |
| *Macrobrachium novaehollandiae* | 15–19 | 101 |
| *Macrobrachium novaehollandiae* | 19–22 | 113 |
| *Macrobrachium rosenbergi* | 22–52 | 26 |
| Gills | | |
| *Macrobrachium novaehollandiae* | 15–19 | 57 |
| *Macrobrachium novaehollandiae* | 19–22 | 94 |
| *Macrobrachium rosenbergi* | 22–52 | 101 |

**Table 2.11** Mean concentrations of copper (µg/g) in the tissues of wild *P. merguiensis* in two locations and *P. monodon* from a prawn farm (from Darmono & Denton 1990)

| Species | Tissue | Location | Copper |
|---|---|---|---|
| *P. merguiensis* | Muscle | TMC | 9.1 |
| | | BRC | 8.6 |
| | Hepatopancreas | TMC | 199 |
| | | BRC | 81 |
| | Gill | TMC | 61 |
| | | BRC | 391 |
| *P. monodon* | Muscle | Prawn farm | 7.2 |
| | Hepatopancreas | Prawn farm | 16 |
| | Gill | Prawn farm | 46 |

TCM: Three Mile Creek; BRC: Bohle River Creek

Shrestha and Morales (1987) found significant seasonal variations in the concentrations of copper among the shrimp *Penaeus brasiliensis* of both sexes from two areas of the Caribbean Sea. Alliot and Frenet-Piron (1990) established a relationship between copper in seawater and metal accumulation in the shrimp *Palaemon serratus* from the La Trinite-sur-Mer Harbour in South Brittany. Summer increases in metal levels in water were reflected in increased metal concentrations in the shrimps. Copper (and other metals) presented a peak of pollution in water every year in July and August, then the metals levels decreased during winter.

Elliott et al (1985a) investigated uptake and depuration of copper and zinc by the barnacle *Eliminius modestus*. During exposure to either metal, uptake of one was accompanied by the loss of the other, ie they acted antagonistically. For this reason the barnacle appeared to be unsuitable as a monitoring organism for copper and zinc in seawater.

## 2.5 Ecotoxicology of copper to gastropods

### 2.5.1 Toxicity

A large number of gastropod species have been used in Australia in pollution studies, including *Thais orbita* and *Morula marginalba* (Wilson et al 1993), *Polinices sordidus* (Hughes et al 1987, Ying et al 1993), *P. incei* (Chapman et al 1985), *Haliotis rubra* (Hyne et al 1992), *Lepsiella vinosa* (Nias et al 1993), *Anadara trapezium* (Scanes 1993), various species of *Conus* (Kohn & Almasi 1993), *Velacumantis australis* and *Pyrazus ebenius* (Ying et al 1993). However, only limited information is available on the lethal and sub-lethal effects of copper on gastropods. One problem with using gastropods is the difficulties associated with the determination of death point. Chapman et al (1985) reported that the 96-h LC50 for copper for *P. incei* was 1.17 mg/L. The criterion for death they used was the failure on the part of the individual to respond to the touch of forceps. Live snails respond to such a stimulus by retracting and closing the operculum. Upon death, muscular control is lost, no such retraction occurs and the animal can be withdrawn from its shell with the forceps. Hughes et al (1987) also reported the 96-h LC50 for copper for *P. sordidus* as 0.77 ± 0.35 mg/L.

Chapman et al (1985) investigated the burying response of *Polinices incei* to copper. They reported that the EC50 calculated after 30 min observation on the burying behaviour of *P. incei* was similar to the EC50 after 96 h in the acute toxicity experiment. They concluded that such a relationship may provide a very rapid way of estimating the lethal effects of pollutants. Similar results were also reported by Hughes et al (1987) on *P. sordidus*. They did notice that one important difference between *P. sordidus* and *P. incei* was the behaviour of the stressed or unburied individuals. *P. sordidus* individuals retracted into their shells with their opercula tightly shut. In *P. incei*, stressed individuals often remained with their feet fully extended, apparently unable to move across the sediment surface or to bury.

### 2.5.2 Bioaccumulation

Batley (1987) examined the distribution and bioavailability of heavy metals in waters and sediments from Lake Macquarie (NSW). Elevated concentrations of zinc, lead, cadmium and copper detected in surface sediments and waters from the northern end of the lake, were attributable to discharges from a lead-zinc smelter on Cockle Creek. The majority of the metals were in a bioavailable form, and were shown to be accumulated in seagrass, seaweeds and bivalves. Also in Lake Macquarie, Scanes (1993) investigated trace metal uptake in the cockle *Anadara trapezium* with the aim of determining whether the concentrations of metals in cockles were related to the concentrations of metals in the surrounding water or sediments. It was concluded that the levels of lead, copper and zinc in the water led to elevated levels in the cockles, irrespective of the concentrations in the surrounding sediment.

Peerzada et al (1990a) collected *Telescopium telescopium* from eleven sites from Darwin Harbour, NT, and analysed for various heavy metals, including copper. The concentration of copper in gastropods ranged from 0.70 to 72.1 µg/g wet weight. High concentrations of copper in *T. telescopium* were found at Race Course Creek, which lies within East Point Reserve and receives treated sewage discharges into the water body close to the sampling location.

## 2.6 Ecotoxicology of copper to bivalve molluscs

### 2.6.1 Toxicity

While bivalve molluscs have been extensively used for monitoring metal levels in temperate waters, few studies have investigated the acute or chronic toxicity of copper to bivalves.

The 96-h LC50 for mussels (*Mytilus edulis)* was reported as 480 µg Cu/L (Amiard-Trignet et al 1986). At copper concentrations above 5 µg/L, an apparently permanent closing of the valves was observed, and at concentrations of 40–80 µg Cu/L, significant mortality (50%) was observed after 14 d exposures (Stromgren 1982). Stromgren (1982) also conducted short term experiments using copper and other metals on the length growth of *Mytilus edulis* and noticed significant reductions of growth rate at 3 µg Cu/L in seawater. The author reported EC50 and EC100 values for growth of 3–4 µg Cu/L and 6–7 µg Cu/L, respectively.

Nell and Holliday (1986) investigated the effects of potassium and copper on the settling rate of larvae of the Sydney rock oyster *Saccostrea commercialis*. A concentration of 200 µg Cu/L increased the settling rate on the first day, but after 5 days, settling rates were lower than the controi and the remaining larvae died.

Nell and Chvojka (1992) studied the effect of bis-tributyltin oxide (TBTO) and copper on the growth of juvenile Sydney rock oysters *Saccostrea commercialis* and Pacific oysters *Crassostrea gigas*. The growth of juvenile (spat) (1.25–2.62 mg dry wt) of the Pacific oyster *C. gigas* and the Sydney rock oyster *S. commercialis* was reduced by only 5 ng/L of TBTO and 16 µg/L of copper, when tested separately. The reduction in growth of the Pacific oysters was more severe than that of the Sydney rock oysters. When Sydney rock oyster spat were exposed to copper and TBTO simultaneously, they suffered a greater reduction in growth than those exposed to only one toxicant. There was no significant interaction ($P>0.05$) between the two toxicants, but an additive effect instead. Copper accumulation in Sydney rock oyster spat increased significantly ($P<0.05$) in the presence of 20 ng TBTO.

Katticaran and Salih (1992) studied the copper-induced metabolic changes (oxygen consumption and lactic acid production) in the marine bivalve *Sunetta scripta*. In 1.0 mg Cu/L exposed clams, siphonal activity and shell valve movements were terminated almost immediately on introduction to the Cu-dosed seawater, and oxygen consumption showed a significant ($P<0.05$) reduction in comparison with clams of the control and 0.3 mg Cu/L dosed groups. The clams exposed to 0.5 mg Cu/L showed a significant ($P<0.05$) reduction in oxygen consumption at 120 and 168 h, compared with the control and 0.3 mg Cu/L exposed clams. After the start of depuration (24 h), oxygen consumption of clams previously exposed to 1.0 mg Cu/L was significantly higher ($P<0.05$) than that of clams previously exposed to lower concentrations and the control. Thereafter at 72, 120 and 168 h of depuration, oxygen consumption of control and experimental clams showed no significant differences. Lactic acid in the adductor muscle of 0.5 mg Cu/L dosed clams showed a significantly higher value (27.3 µg Cu/g FW; $P<0.05$) than control clams (21.6 µg Cu/g ) after 24 h exposure. A statistically significant ($P<0.05$) increase in lactic acid levels was also noticed in the digestive gland (37.8 µg Cu/g) and adductor muscle (29.16 µg Cu/g) of clams previously exposed to 1.0 mg Cu/L, 24 h after the start of depuration (levels in controls being 30.6 µg Cu/g and 22.7 µg Cu/g respectively). At all other sampling times during exposure and recovery, levels of lactic acid in experimental clams were not significantly different from the control.

Krassoi et al (1995) have recently developed a 48-h abnormality bioassay using larvae of the doughboy scallop *Chlamys asperrimus*. Scallop larval development was found to be extremely sensitive to copper, with an EC50 value of 5 µg Cu/L. The lowest observable effect was at 3 µg Cu/L, with no effect at 2 µg Cu/L.

### 2.6.2 Bioaccumulation

Bivalve molluscs have been widely advocated and adopted for monitoring concentrations of metals in the ocean, and certain genera and species, notably oysters and mussels, have been extensively studied in temperate waters (Klumpp & Burdon-Jones 1982). Goldberg (1975) and Goldberg et al (1978) proposed the use of bivalves, especially *Mytilus edulis* in the Mussel Watch Program to monitor levels of heavy metals, initially in the United States and now internationally. Extensive studies have been carried out on the common mussel *Mytilus edulis* (Phillips 1976a, b, 1977a, b, c, 1978a, b, 1979a, Smith et al 1981, Ritz et al 1982, Elliott et al 1985a, 1986, Olafsson 1986). Most of these studies deal with accumulation of heavy metals under varying environmental conditions.

Martincic et al (1992) in an interesting investigation transplanted one-year old cultured mussels, *Mytilus galloprovincialis,* to four different sites (120 individuals per site) throughout the Krka River estuary and the nearby Adriatic coastal area. Concentrations of various metals, including copper, in mussels were analysed four times during 1988/1989. They reported that the body weight of mussels increased and a discernible effect of size on metal concentration was detected. Further, they reported that the fluctuations of copper and cadmium in the adjacent water had no significant effect on their concentrations in the transplanted mussels.

Hung and Han (1992) reported that in Taiwan the first incident of green discoloration in the oysters *Crassostrea gigas* was observed in the Charting coastal area in January 1986 and mortality reports appeared three months later. The cause of green oysters was identified as copper pollution. The copper content of the oysters was extremely high (2100 μg/g and 4400 μg/g dry weight in January 1986 and January 1989, respectively).

In an investigation on metal concentration changes in Pacific oysters, Thomson (1982) reported that Pacific oyster spat (*Crassostrea gigas* Thunberg) transferred from the Tamar Estuary to two growing areas in southern Tasmania were monitored for their metal contents over one growing season (1974–1975). Oysters at Pipeclay Lagoon were grown with stick and tray culture, while those at Dart Island were cultured with the longline technique. Metal content of the oysters increased with time and the trend was similar to the weight-growth curves. Mean dry weights of oysters increased from 0.07 to 1.19 g at Pipeclay Lagoon and from 0.25 to 1.47 g at Dart Island. Metal contents (μg/g) increased at each site, respectively, Fe:57 to 326, 91 to 446; Zn: 269 to 6555, 755 to 5335; Cd: 1.5 to 13.3, 1.9 to 16.3; Cu: 26 to 142, 9 to 116; Pb: 1.9 to 11.9, 0.6 to 3.8. Concentration curves generally showed a downward trend with time. The relationships of metal concentrations with weight did not differ from sample to sample at a site nor did they differ at one site compared with the other. The only exception was lead, which showed no relationship of concentration with weight at Pipeclay Lagoon and a negative one at Dart Island. It is postulated that higher winter concentrations of metals in the oysters were linked with greater solubility of metal ions in lower salinity water.

Thomson (1983) further reported on the short term changes in metal concentrations in cultivated *C. gigas*. He performed two experiments, one of 48 h and the second of 100 h duration, to ascertain changes in metal concentration after flow-through treatments with seawater, filtered seawater and filtered, diluted seawater. Copper was taken up by oysters in low-salinity water with particulates removed.

In an investigation on the cellular metal distribution in the Pacific oyster *Crassostrea gigas*, collected from the metal-rich Derwent Estuary, Tasmania, Thomson et al (1985) reported that all tissues contained elevated copper and zinc concentrations when compared with other bivalves (eg *Mytilus*), with the exception of the reproductive tissues. Calcium, iron, copper and zinc were present in the tertiary lysosomes, and >90% of the total body burden of copper and zinc was accumulated in membrane-limited vesicles of blood amoebocytes.

Talbot (1985) determined ranges of concentrations (mg/kg wet wt) of metals in *S. cucullata* and *Saccostrea* sp (probably *S. commercialis*) from several locations in the Dampier

Archipelago and nearby Cape Lambert. Concentrations of Cu and Zn in individual specimens of these oysters ranged from 1.4 to 555 and from 55 to 1800 mg/kg wet weight, respectively, and reached their maximum values at localised areas adjacent to the Dampier township and iron-ore exporting terminals at Dampier and Cape Lambert. Copper and zinc concentrations correlated significantly with length and wet weight of the oysters.

In another investigation Talbot (1986) sampled the intertidal rock oyster *Saccostrea cuccullata* at eight sites on eight occasions over a 1 year period. Mean Cu and Zn concentrations ranged between 34 and 267 and 206 and 4078 mg/kg dry weight, respectively. In the study area, Cu and Zn emanated from sewage and boat slips (antifouling paints), while Zn probably also originated from coolant water from an electricity power generating station and iron ore exporting facilities. Highest oyster wet weight, Cu and Zn concentrations, and loads occurred in January (spawning period), indicating that metal variation was not reciprocating wet weight. Lowest metal concentrations and loads occurred in October (the period of onset of gametogenesis), while lowest wet weight occurred in April (post-spawning period). No significant ($P<0.001$)) variation in the wet to dry weight ratio was noted temporally. However, significant, though slight, variation was noted between polluted and unpolluted oysters. Results of this study indicated that pollution control monitoring programs should consider: (i) seasonal variation of metal concentrations; (ii) portion of the year during which standards are exceeded; (iii) oyster size and availability for human consumption; (iv) suitability of standards where shellfish are not consumed as a staple diet; (v) appropriate size indices which can be used for selecting specimens for inter-site comparisons; (vi) wet to dry weight calculations, techniques; spatial and temporal variations; and (vii) the physical dynamics of sites used.

From the Georges River estuary, Brown and McPherson (1992) sampled non-commercially grown Sydney rock oysters in spring 1987, and analysed for copper, zinc and lead. The results, when compared with previous data from 1975, indicated a marked increase in the concentration of copper (up to 40%) and zinc (up to 300%). For several sites, the recommended (National Health and Medical Research Council) levels for copper and zinc (70 and 1000 mg/kg wet weight, respectively) were exceeded. There appeared to be a decrease in the concentration of lead since 1975. The gradient of increasing copper and zinc concentrations with increasing distance upstream from the mouth of the estuary reported in 1975 could not be statistically validated. A significant correlation was found between copper and zinc loadings in the oysters. It was noted that data collected in 1975 were based on commercially grown oysters. The use of commercially grown oysters, rather than indigenous oysters to examine interaction of contaminant load and distance upstream, is complicated as commercial oysters are moved within the estuary and between estuaries to maximise growth potential.

Peerzada and Dickson (1989) reported heavy metal concentrations (including copper) in the blacklip oyster *Saccostrea echinata* and the milky oyster *Saccostrea cucullata* from the Arnhem Land coast (Northern Territory) (table 2.12). The same authors (1988) reported copper concentrations of 18–58 ng/g wet weight in oysters from Darwin Harbour. Peerzada and Kozlik (1992) investigated the seasonal variation of heavy metals in oysters from Darwin Harbour (NT). In another investigation Peerzada et al (1990) reported on the distribution of heavy metals in Gove Harbour (NT).

In a recent study, Bou-Olayan et al (1995) investigated the accumulation of lead, cadmium, copper and nickel in the soft tissues of the pearl oyster *Pinctada radiata* from the Kuwait marine environment (three coastal stations during March–June 1990 and March–June 1992). In the 1990 samples, mean metal concentrations in oysters from different sampling stations varied between 0.77 and 1.93 µg/g for Cd, 0.38 and 2.29 µg/g for Cu, 0.44 and 0.64 µg/g for Pb and 0.96 and 1.33 µg/g for Ni. In the 1992 samples, the mean concentrations varied between 1.85 and 3.16 µg/g, 1.01 and 1.18 µg/g, 10.68 and 18.66 µg/g, and 0.94 and 1.33 µg/g for Cd, Cu, Pb and Ni, respectively. The 1992 samples appeared to have significantly ($p<0.01$) higher mean concentrations of Pb, Cd and Cu than the 1990 samples. The difference in patterns of metal occurrence and the significant increase in the Pb, Cd and Cu mean concentrations in the 1992 oyster samples may be due to a contribution from the 1991 Gulf War oil spill.

**Table 2.12** Copper concentration (µg/g wet weight) in oysters from the Arnhem land coast, NT, Australia: range ($x \pm sd$) (from Peerzada & Dickson 1989)

| Location | Date | Cu (µg/g)[a] |
|---|---|---|
| Gove Harbour | June 86 | 22.1–38.2 (29 ± 7.9) |
| Marchinbar Island | June 86 | 6.4–27.8 (14.5 ± 5.6) |
| Raragala Island | June 86 | 1.8–7.7 (5.2 ± 1.9) |
| Coburg Peninsula | June 86 | 6.1–18.6 (9.7 ± 3.5) |
| Rapid Creek, Darwin | Oct 86 | 17.3–39.8 (30.4 ± 7.2) |
| East Point, Darwin | Oct 86 | 45.2–70.2 (58.3 ± 9.4) |
| Channel Island | Oct 86 | 16.8–37.5 (27.0 ± 6.2) |
| Detection limit | | 0.05 |

a National Health and Medical Research Council recommended standard (National Health and Medical Research Council 1979) for copper in molluscs is 70.0 µg/g.

## 2.7 Ecotoxicology of copper to sea urchins

Kobayashi (1980) examined the copper sensitivity of sperm, fertilisation, cleavage, blastula, gastrula, pluteus and metamorphosis stages of a sand dollar (*Peronella japonica*) from Japanese waters and a sea urchin (*Heliocidaris erythrogramma*) from the Pacific coast of Australia. Responses observed included reduction in fertilisation and reduction in development (first cleavage, gastrula, pluteus and metamorphosis). Developmental abnormalities were noted at the fertilisation, 2-cell, gastrula, pluteus and metamorphosis stages. Using effective concentrations of copper at various developmental stages of *P. japonica*, it was found that sensitivity to copper varied from fertilisation to metamorphosis. It appeared that sperm activity was the most sensitive stage, and that fertilisation and gastrulation were more sensitive than first cleavage, blastulation and pluteus formation. Results with *H. erythrogramma* were similar, except that this species was more sensitive to copper at metamorphosis.

Recently, a sperm bioassay using a local species of sea urchin *Heliocidaris tuberculata* has been developed at AWT EnSight in Sydney (1995). Sperm were exposed to copper for 1 h, prior to the addition of the eggs. After a 20 min incubation, percentage fertilisation of the eggs was determined microscopically. The EC50 value for copper was 39.5 µg Cu/L, with 95% confidence limits of ± 3.8 µg Cu/L.

## 2.8 Ecotoxicology of copper to fish

Many factors affect the toxicity of copper to fish, through changes in the availability of copper to the fish (a consequence of the speciation of copper in water), the permeability of the fish to copper (ie the ability of the fish to take up copper from water) and the sensitivity of the fish to the copper taken up (Hodson et al 1979). Whilst extensive work has been done on freshwater species such as rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), bluegill (*Lepomis machochirus*) and fathead minnow (*Pimephales promelas*), little data are available on the toxicity of copper to marine fish.

Early work focused on acute toxicity to adult fish (reviewed by Hodson et al 1979). More recently, early life stages such as embryos and larvae have been shown to be more sensitive to toxicants than adult fish (McKim 1977, Middaugh et al 1994). In addition, sub-lethal toxicity tests with fish have been developed, including tests which measure physiological, biochemical, morphological and genetic changes in fish exposed to specific chemicals (Wong & Dixon 1995). The most common physiological responses used include growth, reproductive success, oxygen consumption, breathing rates, cough response, avoidance behaviour, heart rate and swimming endurance. Biochemical indices such as energy content, the ribonucleic acid (RNA)/deoxyribonucleic acid (DNA) ratio, protein content, or concentrations of specific enzymes or substrates have been suggested as potential short-term, function measures (Giesy et al 1988). The occurrence of lesions, gill deformities and skeletal abnormalities and tumours in marine and freshwater fish from areas of urban development and industrial pollution have also been used (Black 1988).

### 2.8.1 Marine fish

Only limited data are available on the toxicity of metals to various marine fish species. Denton and Burdon-Jones (1986b) carried out acute toxicity tests with six metals, including copper, on adult and juvenile glass perch (*Priopidichtlys marianus*) and juvenile diamond-scaled mullet (*Liza vaigiensis*). They reported that the general order of metal toxicity was Hg> Cu > Cd = Zn > Ni > Pb for total metal concentration. Both adult and juvenile glass perch exhibited similar tolerance towards Hg, Cu and Pb (table 2.13).

The authors also made some preliminary observations of behavioural and physiological changes in adult glass perch when exposed to metals. General symptoms included increased swimming activity which became progressively more erratic and haphazard with time. Gill ventilation rates generally increased from approximately 40 to 110 per minute. Schooling behaviour was disrupted and fish became generally dispersed throughout the tank, whereas controls remained grouped together and were less active. As intoxication progressed, fish became lethargic and often showed complete loss of equilibrium, swimming in head up or head down fashion. There were frequent periods of quiescence interrupted by frenzied, convulsive swimming movements which often terminated in a tetanic coma on the tank bottom.

Lin and Dunson (1993) reported that copper was more toxic than zinc and cadmium to adults of the estuarine fish *Fundulus heteroclitus* and *Rivulus marmoratus*. The 96-h LC50 values for cadmium, copper and zinc at 14 ppt salinity for *Rivulus* were 21.1, 1.4 and 147.9 mg/L respectively, whereas those for *Fundulus* were 18.2, 1.7 and 129.5 mg/L.

Rice et al (1980) found that embryos of the northern anchovy *Engraulis mordax*, were more sensitive than larvae and indicated that, in general, embryos of marine fish are more sensitive than their larvae, whereas larvae of freshwater fish are more sensitive than embryos. Salmon living in the ocean, but spawning in freshwater, behave like saltwater fish in this regard.

**Table 2.13** Acute toxicity of copper to adult and juvenile glass perch and juvenile diamond-scaled mullet (from Denton & Burdon-Jones, 1986b)

| Fish species | Temp:Salinity °C: ‰ | 96-h LC50 (mg/L) | 95% Confidence limit |
|---|---|---|---|
| *P. marianus* (adult) | 20:36 | 2.55 | 2.01–3.24 |
| *P. marianus* (juvenile) | 30:36 | 3.00 | 2.24–4.02 |
| | 30:20 | 4.40 | 2.49–7.79 |
| | 20:36 | 2.10 | 1.40–3.15 |
| | 20:20 | 6.00 | 4.28–8.40 |
| *L vaigiensis* (juvenile) | 20:36 | 2.55 | 1.70–3.83 |
| | 20:20 | 2.65 | 1.61–4.37 |

Blaxter (1977) reported that herring (*Clupea harengus*) eggs had high mortality when incubated in 30 µg Cu/L and those larvae that hatched at this concentration were deformed. Cosson and Martin (1981) investigated the effects of copper on embryos and larvae of *Dicentrarchus laborax* and reported that the hatching rate was reduced at 5 µg Cu/L.

Engel et al (1976) reported that the 4-day LC50 values for larval pinfish (*Lagodon rhomoides*), spot (*Leiostomus xanthurus*), Atlantic croaker (*Micopogon undulantus*) and Atlantic menhaden (*Brevoortia tyrannus*) were 150, 160, 210 and 610 µg Cu/L respectively. Engel and Sunda (1979) compared the sensitivities of embryos of the spot, *Leiostomus xanthurus,* which has planktonic eggs, and the silversides, *Menidia menidia*, which has demersal eggs, to copper concentrations, measured as the free ion in an ion-buffer system. They found that the silversides were more sensitive in terms of hatching success than the spots, since they considered plankotonic eggs to be more delicate than demersal eggs. For the silversides, the greatest toxicity occurred at the time of hatch; the greatest mortality of spot took place prior to hatch.

Swedmark and Granmo (1981) found that when cod *Gadus morhua* were exposed to copper concentrations of 10 µg/L, there was increased mortality of embryos, decreased hatching frequency, prolonged incubation time, and increased abnormalities in the developing cod.

Anderson et al (1991) compared the relative sensitivity of sperm, embryos and larvae from the topsmelt *Atherinops affinis* with copper chloride. The order of sensitivity (most sensitive to least sensitive) was sperm > embryos > larvae, with EC50 values of 109 µg Cu/L (48-h fertilisation), 142–147 µg Cu/L (12 day embryo development) and 238 µg Cu/L (96-h larval mortality). They reported that this fish sperm test provides an attractive alternative to embryo tests for routine toxicity testing because the test is rapid and requires less effort to obtain test organisms.

McNulty et al (1994) investigated the age-specific toxicity of copper to topsmelt larvae aged from 0–20 days post hatch. Growth and survival over 7 days was monitored. Fish aged 0–5 days were less sensitive to copper than fish greater than 7 days old. The LC50 for copper ranged from 365 µg/L in 0-day old larvae to 137 µg/L in 20-day old larvae. The concentration of copper to cause no effect was relatively constant at all ages: 180 µg/L for 1–3 day old larvae and 100 µg/L for all other cohorts.

Powell et al (1981) measured baseline trace element concentrations in eight species of marine fish from Bougainville Island (PNG) . The authors reported that this was the first stage in an assessment of environmental impact associated with mining operations, and concluded that in general, concentrations of Cu, Pb, Zn, Cd, Hg and As in edible portions of the fish complied with Australian National Health and Medical Research Council public health standards.

### 2.8.2 Freshwater fish

*Toxicity*

The acute toxicity of copper to a range of freshwater fish in soft and hard waters is shown in table 2.14. Some freshwater fish, especially salmonoids, are much more sensitive to copper than marine fish, with 4-day LC50 values of 40-80 µg Cu/L in softwaters. Increased water hardness dramatically reduces copper toxicity as a result of the higher concentrations of carbonate and bicarbonate in hard waters complexing copper and converting it to non-toxic forms. High pH, phosphate and organic matter also lower copper toxicity.

**Table 2.14** Acute toxicity of copper to freshwater fish

| Fish species | 4-day LC50 (µg Cu/L) | |
|---|---|---|
| | Softwater | Hardwater |
| Rainbow trout | 50 | 250 |
| Brook trout | 35 | 150 |
| Atlantic salmon | 50 | 250 |
| Coho salmon | 50 | 250 |
| Fathead minnow | 100 | 800 |
| Goldfish | 50 | 650 |
| Carp | 250 | 3000 |

Even at very low concentrations, copper can affect the growth, spawning frequency, egg production and hatching, appetite and avoidance behaviour of freshwater fish (table 2.15; Hodson et al 1979). Water hardness does not have as much effect on sub-lethal toxicity of copper as it does on acute toxicity.

Pilgaard et al (1994) exposed the rainbow trout, *Oncorhynchus mykiss,* to a sub-lethal copper concentration of 0.5 mg/L for nine days in normoxic ($pO_2$ > 130 mmHg) and hypoxic (70 mm Hg) hard water. The authors reported that there were no signs of respiratory dysfunction in any of the exposed fish. The concentration of copper increased in mucus, gill liver and kidney tissues, whereas the copper concentrations of white muscle, heart, spleen and whole body were only marginally affected by copper exposure.

De Boeck et al (1995) measured oxygen consumption and ammonia excretion in the common carp *Cyprinus carpio* (15–30 g body weight) after exposing them to copper levels of 0.22, 0.34 and 0.84 µmol/L for two weeks. Oxygen consumption dropped significantly after exposure to 0.34 and 0.84 µmol Cu/L, whereas nitrogen excretion remained stable. After one week of continuous exposure to 0.34 µmol Cu/L, the oxygen consumption showed an apparent recovery, while the ammonia quotient (mole to mole ratio of ammonia excreted to oxygen consumed) did not. The authors reported that at a copper concentration of 0.84 µmol/L no recovery was observed. They also suggested that measurements of oxygen consumption in combination with nitrogen excretion measurements can be useful indicators of stress.

**Table 2.15** Sub-lethal effects of copper on freshwater fish

| Fish species | Response | LOEC (µg/L) |
|---|---|---|
| Rainbow trout | avoidance behaviour | 4 |
| Atlantic salmon | appetite | 6 |
| Brook trout | growth | 6 |
| Goldfish | behaviour | 10 |
| Fathead minnow | growth | 15 |
| Bluegill | reproduction | 30 |

Castano et al (1995) carried out acute toxicity tests using selected metals including copper and phenols on RTG2 and CHSE 214 fish cell lines. RTG2 was derived from the gonad of the rainbow trout (*Oncorhynchus mykiss*), and CHSE 214 from an embrionary cell line derived from the chinook salmon *O. tshawytscha*. They measured three endpoints: protein, cell viability (measured by the uptake of Neutral Red stain) and intracellular ATP content. The 48-h EC50 values for copper (µg/mL, with 95% confidence limits) for these three endpoints were 6.3 (5.8–7.8) and 12.7 (9.7–16.7) for protein, 6.3 (5.5–7.3) and 9.5 (7.7–11.7) for cell viability and 6.5(5.9-6.8) and 11.9(10.0-14.1) for ATP for the CHSE and RTG2 cell lines respectively. They concluded that *in vitro* cell cultures were a suitable tool for assessing the toxicity of different chemicals, as they could be performed more easily and more rapidly than 96-h acute tests with whole fish.

James and Sampath (1995) studied the individual and combined effects of copper and ammonia on some biochemical and physiological parameters in the catfish *Heteropneustes fossilis*. The 96-h LC50 value obtained for copper was 2.4 mg/L (95% confidence limits of 1.72–3.35). Their results revealed that copper was more toxic than ammonia, but copper and ammonia together were more toxic than the individual toxic effects. They reported concentration-dependent significant ($r = -0.993$; $p<0.01$) reductions of glycogen content in gill, liver and muscle, with a concomitant increase in blood glucose concentrations.

*Mechanism of toxicity*

Histopathological examination of fish exposed to high dissolved copper concentrations showed pathological changes in the gills, kidneys, liver and haematopoietic tissue (Baker 1969). Copper inhibits a range of enzymes in fish, including alkaline phosphatase, acid phosphatase, xanthine oxidase and catalase (Jackmin et al 1970) and might also affect osmoregulation. Sockeye salmon showed increased levels of cortisone and total corticosteroids in plasma when they were exposed for only two hours to water containing 6.5 µg Cu/L (Donaldson & Dye 1975). This hormonal response shows that copper must occur at the molecular level, probably involving the reaction of copper ions with sulfhydryl groups in enzymes and proteins.

*Bioaccumulation*

Smith and Morris (1992) reported on the impacts of the Ok Tedi Copper Mine waste on the fish assemblages of the Lower Ok Tedi and Middle Fly Rivers, Papua New Guinea. The period of operation has been characterised by increasing suspended solids levels with concomitant increases in dissolved and particulate copper. During gold production, some residual cyanides were released into the river system, which were predominantly associated with copper complexes of low toxicity. The fish assemblages at two sites in the Ok Tedi and one in the Fly River were examined. Mine operations rapidly reduced fish stocks at Ningerum, the site closest to the mine. At the Fly River site, the fish stocks were greater and more diverse, and the changes in the fish assemblages were more gradual.

Kyle (1988) analysed zinc, copper, lead and cadmium in fish from the Ok Tedi tributary prior to the onset of mining. Concentrations were determined for whole specimens rather than the flesh alone, since whole fish are consumed in this region. Three species were analysed during the study and the results were compared with data for other species examined during the course of the Ok Tedi Environmental Study. Variation within species was found to be greater than variation between species, and the order of concentration was found to be zinc > copper > lead and cadmium. Concentrations of cadmium were found to exceed the Australian National Health Standards, whilst those for copper, lead and zinc were below these standards.

Pelgrom et al (1994) exposed juveniles of tilapia (*Oreochromis mossambicus*) for 96-h to various sub-lethal concentrations of copper or cadmium under both fed and non-fed conditions. Exposure to one metal not only resulted in an increased whole body content of that metal, but also influenced the concentrations of the other metals present in the fish. Further, the total amount of copper and cadmium accumulated during exposure to heavy metals was influenced by the nutritional state of the fish.

## 2.9 Summary

Copper is essential to life for both humans and aquatic animals. However, after mercury and silver, ionic copper is the most toxic metal species to a wide range of aquatic organisms. Copper is often present in significant concentrations in leachates of mine tailings due to bacterial oxidation of pyrites, which results in the production of sulphuric acid and dissolution of copper.

The toxicity of copper is critically dependent on the speciation of copper, ie its physicochemical form. The most toxic copper species are the free metal ion ($Cu^{2+}$) and some lipid-soluble copper complexes (eg copper ethylxanthate). Particulate copper, and copper bound by humic, fulvic and tannic acids present in natural organic matter in waters, have very low toxicity. It is therefore important to understand the speciation of copper, because a water body with quite high total copper concentration might actually have little effect on aquatic life if the dissolved organic carbon (DOC) concentration in the water is high. The copper complexing capacity of a water is a measure of the ability of the natural complexing agents in the water to detoxify copper.

Unpolluted coastal seawater contains 0.3–1 μg/L of total dissolved copper, while pristine freshwater rivers typically have 0.33 μg/L of copper. The Australian and New Zealand Environment and Conservation Council (ANZECC) places a limit of 5 μg/L total copper in seawater, and 25 μg/L copper in freshwaters, depending on water hardness. Regulatory bodies such as ANZECC do not take the speciation of copper into account in setting their limiting criteria.

Copper is highly toxic to some species of algae, which are the start of the food chain. Total copper concentrations as low as 5 μg/L (or $3 \times 10^{-11}$ M free cupric ion activity) will affect the cell division rate of some algae. For this reason, algal bioassays are often used to provide the 'worst case scenario' for copper toxicity.

Total dissolved copper concentrations of 50–200 μg/L will change the behaviour of sea anemones, and some corals are affected by copper concentrations as low as 10 μg/L. Various species of crustacean (prawns, crabs, amphipods etc) are also sensitive to copper, with acute (ie short-term exposure of 14 days) LC50 values (copper concentration required to kill 50% of the test animals in a fixed period) of 100–1000 μg/L. For these animals, the chronic (ie long term exposure, typically 30 days) LC50 values are 100–300 μg Cu/L and sub-lethal effects (eg hatching rate of embryos) can occur at concentrations as low as 10 μg/L. The toxicity of copper to prawns increases with increasing temperature and with decreasing salinity.

Gastropods (eg snails) and bivalve molluscs (oysters, mussels etc ) are more tolerant of copper and can accumulate quite high concentrations of the metal without ill effects. Some species of snails were found to have 4-day LC50 values of 0.8–1.2 mg/L copper, and certain species of snails collected in Darwin Harbour accumulated 0.7–72 μg/g copper. Oysters can accumulate as much as 4000 μg/g copper dry weight, and these levels cause a green colouration in the tissue. Bivalve molluscs react to high concentrations of copper in water by closing their valves and ceasing pumping. Mussels stop pumping when copper in water exceeds 5 µg/L and they have a 14-day LC50 of 40–80 µg/Cu L. The growth rate of mussels is reduced at 3 µg/L copper in water.

Marine fish appear to be relatively tolerant of copper, although few data are available. The 4-day LC50 copper values for Australian juvenile glass perch and diamond-scaled mullet were reported as 2000–6000 µg/L. Some freshwater fish species, especially salmonoids (salmon, trout), are much more sensitive to copper than marine fish. The toxicity of copper to freshwater fish increases with decreasing water hardness, and in soft waters the 4-day LC50 values for salmonoids are typically 40–80 µg/L copper. Coarse fish (eg carp) are more resistant to copper, with 4-day LC50 values of 1000–3000 µg/L copper. The hatching rates of the eggs of Atlantic salmon and rainbow trout are reduced at copper concentrations of 10 and 20 µg/L respectively.

More research is needed on the physicochemical speciation of copper in natural and polluted waters and sediments, and on the effect of copper speciation on toxicity to aquatic organisms. There is a lack of information on the effects of copper on Australian marine fish and on benthic (sediment dwelling) organisms.

# 3 Experimental

## 3.1 General analytical procedures

The following general analytical procedures were used. All acids were of ultra-pure grade (Normaton) and all water was high purity (Milli-Q). All other reagents were analytical grade. All bottles (Nalgene) used in the sampling program were made from low density polyethylene and were pre-washed in Extran 300 detergent, followed by two washes in 10% (v/v) nitric acid. Bottles were rinsed thoroughly with Milli-Q water prior to bagging in sealable plastic bags in a Class-100 clean room.

## 3.2 Collection of Macquarie Harbour water samples

Macquarie Harbour mid-water samples (15–20‰ salinity) from 6 to 9.5 m depth were collected on 12–13 October 1995, from eight sites (fig 3.1). The site numbers correspond to the Tasmanian Department of Environment and Land Management's (DELM) routine water monitoring stations. Salinity measurements with a Hydrolab Water Quality Meter (supplied by DELM) were used to determine the depth at which to collect the mid-salinity waters. Five litres of each sample, collected in oceanographic sampling bottles, were dispensed into polyethylene containers. A 100 mL subsample was retained for ASV-labile copper determinations at DELM. Temperature, salinity, pH, turbidity, dissolved oxygen and redox potential were determined at the same time using the Hydrolab Water Quality Meter.

Within 48 h of collection, the water samples were filtered through a 0.45 µm membrane filter using an acid-washed plastic membrane filter apparatus. Each sample was split into three parts:

1. 100 mL was frozen immediately for ortho-phosphorus analysis at DELM, Hobart.
2. 500 mL was acidified with 2 mL of concentrated nitric acid, chilled and retained for analysis of total dissolved metals at the CSIRO Centre for Advanced Analytical Chemistry (CAAC), Sydney.
3. 4 L was chilled and retained for DOC, pH, ASV-labile copper and algal toxicity tests at CSIRO CAAC.

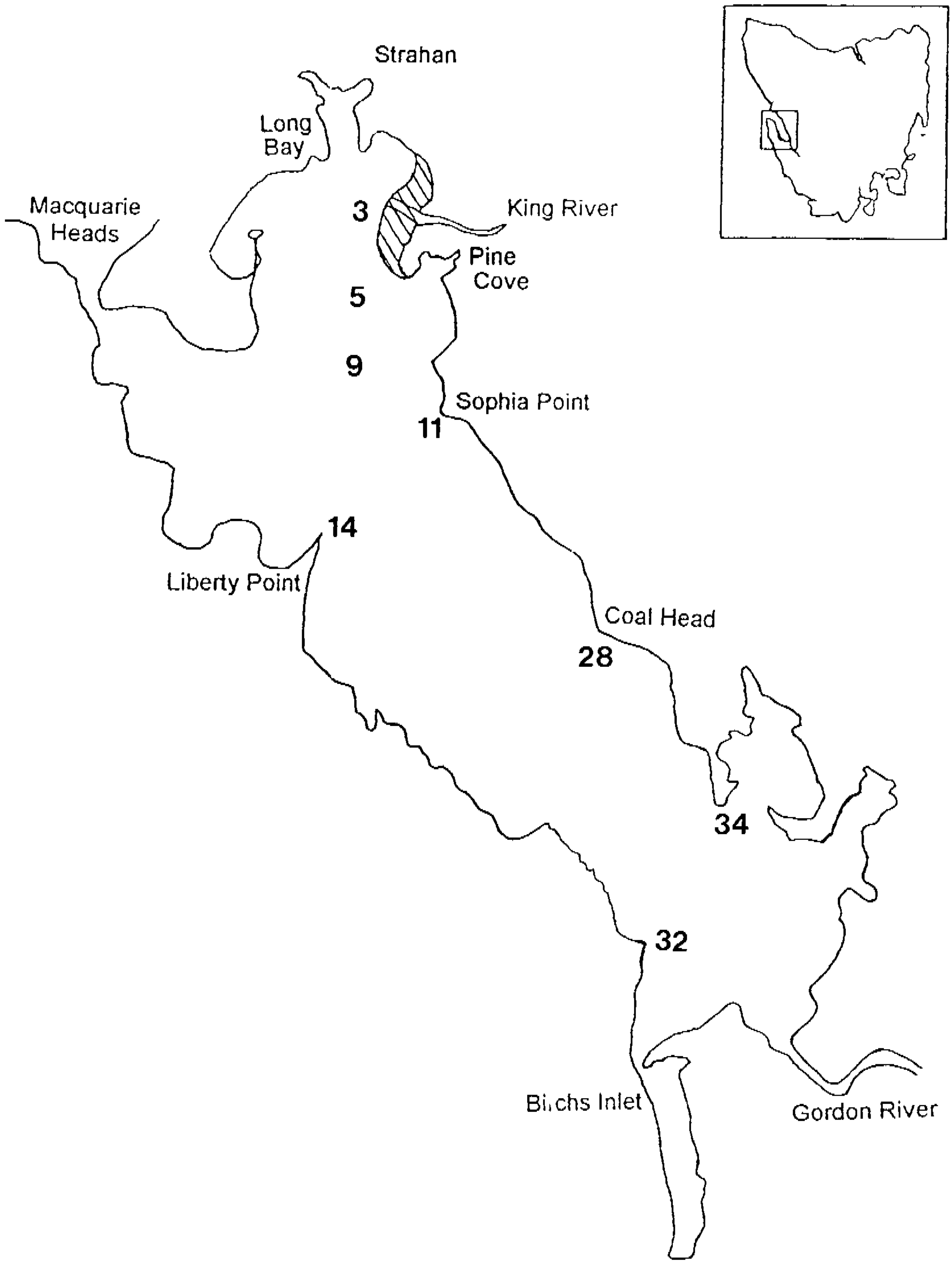



**Figure 3.1** Field collection sites in Macquarie Harbour

A second sampling trip was organised on 14 December 1995 to collect large volumes of Macquarie Harbour water for the invertebrate and fish toxicity tests. Measurements of salinity, pH, temperature, dissolved oxygen, turbidity and redox potential were taken at the same time as sample collection. Mid-depth water was pumped into four fibreglass tanks (each with 1800 L capacity). Water was collected from station 9 and station 14 (near the west coast fish farms) as these sites had been shown to have high dissolved copper levels during the October sampling. The water quality characteristics of the Macquarie Harbour samples collected in October and December are shown in table 3.1.

Sub-samples (200 L) for fish toxicity testing were sent to the Department of Aquaculture, University of Tasmania, Launceston. The larger tanks were transported overland to Devonport, across Bass Strait by ship and by truck from Melbourne to Sydney by the Commonwealth Department of Administrative Services. The tanks arrived in Sydney approximately one week after water collection. Subsamples (5 L) were immediately filtered for the algal bioassays and chemical analyses as above. For the amphipod and fish bioassays, unfiltered water was used.

**Table 3.1** Water quality characteristics for Macquarie Harbour samples collected in October and December 1995

| Station no | Depth (m) | Water temperature (°C) | Salinity (‰) | pH |
|---|---|---|---|---|
| October sampling | | | | |
| 2 | 8 | 11.7 | 18 | 7.5 |
| 5 | 7 | 11.6 | 18 | 7.5 |
| 9 | 6 | 11.6 | 18 | 7.5 |
| 11 | 9.4 | 11.6 | 15 | 7.8 |
| 14 | 9.5 | 11.7 | 18 | 7.5 |
| 28 | 9.5 | 11.6 | 18 | 7.6 |
| 32 | 8.5 | 11.7 | 18 | 7.5 |
| 34 | 9.1 | 11.7 | 18 | 7.6 |
| December sampling | | | | |
| 9 | - | 14.2 | 21 | 7.9 |
| 14 | - | 14.1 | 20 | 7.7 |

## 3.3 Chemical analyses

### 3.3.1 Metals

Total copper in the unfiltered samples and total dissolved copper in the filtered samples were determined by graphite furnace atomic absorption spectroscopy (GFAAS) at DELM, Hobart. Dissolved copper, aluminium, chromium, manganese, iron, cobalt, nickel, zinc, silver, barium, cadmium and lead were determined by inductively coupled plasma atomic emission spectroscopy (ICPAES) at CAAC, Sydney.

Complexing capacity and labile copper was determined using anodic stripping voltammetry. Sample aliquots (20 mL) were pipetted into PTFE polarographic cells, spiked with additions of copper (0–50 µg/L), covered and allowed to equilibrate overnight. Following equilibration, 40 µL of 5 M sodium nitrate was added. Differential pulse anodic stripping voltammetry (DPASV) measurements were made using a Metrohm 646 Voltammetric Analyser with a hanging mercury drop electrode. Each sample was stirred and purged with nitrogen for 300 seconds, then a new mercury drop was formed and deposition carried out for 300 s at -0.6V (vs Ag/AgCl). After 20 s without stirring, a potential scan was initiated (scan rate 3.3 mV/s, pulse height 50 mV, pulse step 2 mV) and the copper oxidation wave recorded between -0.2 and 0.2 V. The resulting titration plots were used to calculate ASV-labile copper (y-axis intercept) and complexing capacity (extrapolation of the linear portion of the curve to the x axis) of the sample (Apte & Day 1993).

### 3.3.2 Dissolved organic carbon (DOC)

DOC in each water sample was determined by photocatalytic oxidation in an ANATOC analyser (SGE Instruments). The samples were first adjusted to a pH of 3.5 and sparged with air for 5 minutes to remove dissolved inorganic carbon.

### 3.3.3 Nutrients

Oxidised nitrogen and ortho-phosphate were determined on each sample using standard methods (APHA 1989).

## 3.4 Toxicity tests

Water samples from eight sites in Macquarie Harbour (collected in October, 1995) were screened for toxicity to the alga *Nitzschia closterium*. In addition, water samples from station 9 and 14 (collected in December, 1995) were assessed for toxicity using the *Nitzschia* growth inhibition test, the algal enzyme inhibition test, the amphipod growth inhibition test and the juvenile flounder bioassay.

### 3.4.1 Algal growth inhibition bioassays

Algal growth inhibition bioassays using both filtered and unfiltered Macquarie Harbour water samples were carried out at CSIRO CAAC, Sydney. Water samples collected in October, 1995 from eight sites in Macquarie Harbour (station 3, 5, 9, 11, 14, 28, 32 and 34) were screened for toxicity to the alga *Nitzschia closterium*. In addition, the toxicity of station 9 and 14 waters (collected in December, 1995) to *Nitzschia* were assessed at the same time as the bioassays with the amphipod and flounder. The complexing capacity of these two samples was also determined by spiking filtered station 9 water and unfiltered station 14 water with increasing amounts of ionic copper.

In addition to the Macquarie Harbour waters, the toxicity of ionic copper to *Nitzschia closterium* over 72 h in clean dilution water (at a range of salinities to match the Macquarie Harbour waters) was also determined.

*Algal stock cultures*

The unicellular estuarine diatom, *Nitzschia closterium* (Ehrenberg) W. Smith (Strain CS 5), was originally isolated from Port Hacking, NSW. The diatom was cultured in medium f (Guillard & Ryther 1962) with the iron and trace element concentrations halved. The culture was maintained axenically on a 12 h light:12 h dark cycle (Philips TL 40 W fluorescent daylight, 4500 lux) at 18°C.

Cells in log phase growth were used in the algal bioassays according to the standard protocol (Stauber et al 1994). The inoculum was washed and centrifuged three times prior to use in the algal assays, to remove culture medium.

*General algal bioassay procedure*

A summary of the bioassay conditions is given in table 3.2. Controls were prepared by diluting natural 0.45 µm filtered seawater (collected form Port Hacking, NSW) with Milli-Q water to exactly the same salinity as each of the Macquarie Harbour water samples (15–20 ‰). Fifty millilitres of control water were dispensed into 2 to 3 glass Erlenmeyer flasks, which had been pre-silanised with Coatasil (BDH) to prevent copper adsorption to the flask walls.

A dilution series of each Macquarie Harbour water sample was prepared with salinity-matched seawater and 50 mL of each dilution dispensed into silanised flasks. Two to three replicates at each of 2 to 5 concentrations were used. To each flask, 0.5 mL of 26 mM sodium nitrate and 0.5 mL of 1.3 mM potassium dihydrogen phosphate were added. Sub-samples were taken from each flask (5 mL) and acidified prior to determination of total dissolved copper by ICPAES. For the ionic copper calibration bioassays, 2 to 3 replicates at each of 6 copper concentrations (2.5–80 µg Cu/L) were prepared in the same way.

**Table 3.2** Summary of the test protocol for the *Nitzschia closterium* growth inhibition bioassay

| | |
|---|---|
| Test type | Static |
| Temperature | 18 ± 1°C |
| Light quality | Daylight Fluorescent Lighting |
| Light intensity | 14000 lux |
| Photoperiod | 12 hour light : 12 hour dark |
| Test chamber size | 200 mL |
| Test solution volume | 50 mL |
| Renewal of test solutions | None |
| Age of test organisms | 5–6 days |
| Initial cell density in test chambers | 2 – 4 x $10^4$ cells/mL |
| No. of replicate chambers/concentration | 2–3 |
| Shaking rate | Once daily by hand |
| Dilution water | Natural 0.45 μm filtered seawater |
| Effluent concentrations | Minimum of 5 and a control |
| Dilution factor | 0.3 or 0.5 |
| Test duration | 72 h |
| Endpoint | Growth (cell division) |
| Test acceptability | Control cell division rates 1.1 ± 0.3 doublings per day |
| Effluent volume required | <500 mL |

Each flask was inoculated with 2–5 x $10^4$ cells/mL of a prewashed *Nitzschia* suspension. Flasks were incubated at 18°C on a 12:12 h light/dark cycle at 14000 lux. Cell density in the algal bioassays was determined daily for three days using a Coulter Multisizer II Particle Analyser with 70 μm aperture. Aliquots of cells from each flask were homogenised in a tissue grinder to break cell clumps prior to counting. A background particle count (dilution water without cells) was subtracted from all Coulter counts.

At the end of each experiment, subsamples were taken for pH measurement and copper analysis by ICPAES.

A regression line was fitted to a plot of $\log_{10}$ cell density versus time (h) for each flask and the cell division rate (growth rate) per hour (μ) determined from the slope. Cell division rates per day (3.32 x μ x 24) were calculated for each treatment and controls. The bioassay was acceptable if the cell division rate in the controls was 1.1 ± 0.3 divisions per day (at 20‰ salinity).

*Statistical analyses*

The 72-h EC50 was calculated using Microtox software, trimmed Spearman Karber or Probit analysis. After testing the data for normality and homogeneity of variance, Dunnett's Multiple Comparison Test was used to determine which concentrations were significantly different to the controls. This enabled estimation of the LOEC (the lowest concentration to cause a significant effect on algal growth) and NOEC (the highest concentration to cause no significant effect on algal growth compared with the controls).

*Complexing capacity experiments*

The complexing capacity of two Macquarie Harbour waters (collected in December, 1995) was determined by spiking filtered station 9 water and unfiltered station 14 water with increasing amounts of ionic copper (5–80 μg Cu/L). Each flask was equilibrated overnight prior to the addition of the *Nitzschia* inoculum and the bioassay carried out according to the standard procedure. The EC15 value (ie the concentration of copper which reduced the growth rate by 15% compared with the controls) was equivalent to the complexing capacity of the sample. Complexing capacity is a measure of the total concentration of all metal binding sites in the solution and gives an estimate of how much more metal can be added before free metal ion appears and causes toxic effects.

### 3.4.2 Algal enzyme inhibition bioassays

A summary of the test protocol is given in table 3.3.

*Algal stock cultures*

*Dunaliella tertiolecta* Butcher (Stain CS-175) was originally obtained from the CSIRO Division of Fisheries Phytoplankton Culture Collection, Hobart. The alga was cultured axenically in a modified half-strength medium f, with the iron and trace element concentrations halved. The culture was maintained axenically on a 12 h light:12 h dark cycle (Philips TL 40 W fluorescent daylight, 4500 lux) at 18°C.

Cells in log phase growth were used in the algal bioassays according to the standard protocol (Stauber et al 1995; Peterson & Stauber 1996). The inoculum was washed and centrifuged three times prior to use in the algal assays, to remove culture medium. Washed *Dunaliella* cells were counted using a Coulter Multisizer II Particle Analyser with 70 μm aperture, to provide an inoculum so that the final cell concentration in each assay tube was $10^5$ cells/mL.

*Bioassay*

The fluorescent substrate MU-gal was prepared by weighing 0.0625 g of MU-gal into a small beaker. Eight millilitres of dimethylformamide (DMF) was added and the solution sonicated to dissolve the substrate prior to the addition of 8 mL of Milli-Q water. The solution was passed through a Waters Accell Plus QMA Sep-Pak Plus cartridge (pre-rinsed with 20 mL of 0.5 M NaOH and 40 mL of Milli-Q water) at a flow rate of 4 mL/min. The Sep-Pak was then rinsed with 8 mL of Milli-Q water and the eluates combined and made up to 25 mL with Milli-Q water. The solution was filter-sterilised through a 0.22 μm membrane filter and used immediately in the enzyme assay.

A dilution series of Macquarie Harbour water or ionic copper (2.5–40 μg Cu/L) was prepared in salinity-matched seawater. Three to five replicates at each concentration were prepared, together with 3 to 5 replicate controls, by pipetting 9.1 mL of each dilution into sterile borosilicate glass culture tubes (16x100mm) with polypropylene screw caps. To each tube, 0.5 mL of filter-sterilised 1 M PIPES buffer (pH 7.2), 0.4 mL of MU-gal and $10^6$ algal cells were added. The tubes were incubated for 60 minutes at 44.5°C. After cooling, 0.4 mL of a carbonate base stock solution (2.8 g sodium carbonate, 1.2 g sodium hydrogen carbonate and 10 g sodium citrate in 100 mL Milli-Q water) was added to convert the MU to its most fluorescent form.

Fluorescence was determined using a Perkin-Elmer LS-5 Luminescence Spectrometer, with an excitation wavelength of 375 nm and emission wavelength of 465 nm. The fluorimeter was pre-calibrated with methylumbelliferone standards (0, 20, 50 and 80 nM) and the fluorescence response reported as concentrations of MU.

Toxicity was determined by the reduction in fluorescence in the presence of toxicant compared with the controls. Fluorescence response was corrected for chemical hydrolysis of the MU-gal substrate alone, fluorescence of algae in the absence of MU-gal and fluorescence of the water sample alone (± MU-gal). Fluorescence inhibition was calculated as a percentage of the control response. Statistical analyses were as for the growth inhibition bioassays.

**Table 3.3** Summary of the test protocol for the marine algal (*Dunaliella tertiolecta*) enzyme inhibition test

| | |
|---|---|
| Test type | Static |
| Temperature | 44.5 ± 0.5°C |
| Test chamber size | 12 mL |
| Test solution volume | 10 mL |
| Renewal of test solutions | None |
| Age of test organisms | 5–9 days |
| Initial cell density in test chambers | 1 x $10^5$ cells/mL |
| No. replicate chambers/concentration | 3–5 |
| Dilution water | Natural 0.22 µm filtered or autoclaved seawater |
| Effluent concentrations | Minimum of 5 and a control |
| Dilution factor | 0.3 or 0.5 |
| Test duration | 1–24 hours |
| Endpoint | Enzyme activity (fluorescence) |
| Test acceptability | Control response: 260–1223 nM MU |
| Effluent volume required | <500 mL |

### 3.4.3 Amphipod growth bioassays

Stocks of laboratory cultured *Allorchestes compressa* Dana and its food and habitat, the seagrass *Heterozostera tasmanica* were obtained from the Marine and Freshwater Resources Institute, Queenscliff, Victoria. They were maintained in the laboratory in flowing seawater in 70 L glass tanks at 19 ± 1°C.

First instar juveniles were collected from female marsupia after brief exposure to anaesthetic (0.1% MS222, Sandoz). They were kept in clean seawater for 24 h prior to use in the bioassays.

Bioassays were carried out in 10 L acid-washed glass aquaria receiving a constant input of medium and held at a constant water level using an outlet siphon system. Dilution water was prepared by adjusting the salinity of clean seawater to 21‰ with deionised water. Four different dilutions of Macquarie Harbour water (station 9 and 14) were prepared by diluting the water samples with this 21‰ salinity seawater—100%, 50%, 25% and 12.5%. Each dilution, together with controls, had 4 replicates. Premixed dilutions were prepared and stored in 220 L drums and dispensed by Masterflex pumps to all tanks within a treatment. Thirty grams of rotten seagrass was added to each tank as food and habitat for the juveniles. After 2 days, 30 juveniles were added to each tank. Water quality parameters including temperature, pH, dissolved oxygen and salinity were measured daily in each tank. Concentrations of total copper in each tank were measured initially and once each week for the duration of the experiment (27 days).

At the end of the 27-day exposure period, all surviving amphipods were recovered and counted. They were kept in deionised water for 2–3 h, before drying in pre-weighed crucibles at 60°C. The crucibles were then weighed on a Sartorius analytical balance to calculate the mass of amphipods per tank.

An additional experiment with ionic copper (added as copper sulphate) was carried out. Nominal copper concentrations of 3, 6, 12, 25 and 50 µg/L, together with controls, were prepared in clean 21‰ salinity seawater, with 4 replicates per treatment. The experimental procedure was the same as that described for the Macquarie Harbour waters.

### 3.4.4 Juvenile flounder bioassays

*Bioassay*

Juvenile flounder (70 days old) ranging in length from 9 mm to 22 mm (mean 15.03 mm) and an average weight of 0.12 g were obtained from the Marine Research Laboratories hatchery. Fifteen fish were randomly assigned to each 0.5 L plastic container. As the fish were cultured in full salinity seawater they were gradually acclimated to 20‰ salinity over a 48 h period.

At the end of the acclimation period the containers were randomly assigned to different treatments. The following treatments were tested: control, 100% water from Macquarie Harbour (station 9), 50% water from Macquarie Harbour (station 9), 100% water from Macquarie Harbour (station 14), 50% water from Macquarie Harbour (station 14), and ionic copper (5–160 µg/L). These nominal concentrations were prepared using stock solutions of copper sulphate and filtered control water from the Marine Research Laboratories hatchery water supply from Storm Bay, Taroona. There were 4 replicates per treatment, each of which was adjusted to 20‰ salinity. The test was run as a semistatic test for fourteen days with the water gently aerated and exchanged every 24 hours. The temperature was maintained in a controlled temperature room at 16°C.

At the time of daily water exchange, the bottom of the containers was cleaned of faeces and uneaten food and any dead or moribund fish removed. The fish were fed twice a day with newly hatched *Artemia* and feeding was stopped 24 h before the end of the experiment. Water quality was monitored at least once a day. Dissolved oxygen levels ranged from 4.3 to 8.0 mg/L (with a mean at the end of 24 h of 6.9 ± 0.7 mg/L and a mean at the beginning of 24 h of 6.7 ± 0.4 mg/L) and the temperature ranged from 14.3°C to 16.5°C (with a mean at the end of 24 h of 15.2 ± 0.4°C, and a mean at the beginning of 24 h of 15.9 ± 0.4°C).

At the end of the 14-day exposure, 10 fish were removed from each container. These fish were anaesthetised with an overdose of benzocaine and either fixed for histology or frozen for copper analysis. The remaining 5 fish left in each container were challenged with fresh water to assess the effect of exposure to copper on osmoregulation.

*Histology*

The fish were processed using routine histopathological procedures. After embedding in paraffin, 4 mm sections were cut and stained using hematoxylin-eosin. PAS (Periodic Acid Schiff staining method) was used to identify glycogen in liver cells. Slides were then examined under the microscope at 400x magnification.

*Copper analysis*

Individual fish were freeze-dried (DynaVac Mini Ultra Cold) to constant mass. Approximately 50 ±10 mg of freeze-dried fish was weighed accurately into acid-washed 25 mL Erlenmeyer flasks. Where individual fish weighed less than 40 mg, two or more fish from the batch were composited. Two samples were analysed from each container, to give eight samples per treatment.

One millilitre of AR grade $HNO_3$ was added to the Erlenmeyer flask and a watch glass was placed over the mouth of the flask. After overnight digestion, the samples were heated to reflux for 1.5 to 2.0 h on a hotplate until the evolution of oxides of nitrogen ceased. AR grade $H_2O_2$ (1 mL) was added to the hot digest in 0.2 mL aliquots, and the solution was then cooled to room temperature. Nitric acid (1 mL of 10%) was added and the flask warmed gently for 15 minutes. The samples were cooled to room temperature and the digestate transferred to acid-washed polypropylene tubes which were made up to 10 mL with deionised water. The samples were then analysed by graphite furnace AAS (Varian Spectra AA 300) which was calibrated with a matrix-matched standard and blank. At least three blanks and at least one Standard Reference Material (Community Bureau of Reference BCR No 357, Certified Reference Material CRM278 mussel tissue) was carried through with each batch of samples.

*Freshwater challenge*

Five survivors from each container were exposed to fresh water (aged and aerated tap water) for 24 h to assess the effects of exposure to copper on osmoregulation of flounder.

*Statistical analysis*

Statistical analyses were as for the algal and amphipod bioassays. In addition, the histology results were analysed on the basis of prevalence, using one factor ANOVA (11 levels) and each container being a replicate (4 in each treatment). If the results of ANOVA were significant, the statistically different treatments were identified using a-posteriori multiple comparison of means SNK test (Student-Neuman-Keuls test).

### 3.5 Risk assessment

Details of the risk assessment methodology are given in Appendix A.

## 4 Results and discussion

### 4.1 Trace metals in Macquarie Harbour waters

Total dissolved metals in Macquarie Harbour waters collected in October and December, 1995 for use in the bioassay testing program are shown in table 4.1. Dissolved copper ranged from 26 to 42 µg/L (October sampling), with the highest concentrations at stations 9, 11, 5 and 3 immediately below the outflow of the King River. Dissolved copper concentrations in water from station 9 and 14 in the December sampling were much lower (10 and 12 µg/L respectively). The dissolved copper concentrations were much higher than the ANZECC limits for total copper in seawater (5 µg/L). Concentrations of other metals including manganese, iron, aluminium and zinc were also elevated above typical seawater background concentrations. Cadmium, lead, cobalt, silver and chromium were below detection limits by ICPAES. Teasdale et al (1996) found that dissolved copper in Macquarie Harbour waters correlated highly with dissolved manganese concentrations ($r^2$=0.97), suggesting that dissolved copper was largely associated with colloids high in manganese (probably combined with iron) in the Harbour.

Total copper, dissolved copper, ASV-labile copper and dissolved organic carbon (DOC) in Macquarie Harbour waters are shown in table 4.2. In general there was agreement between measurements of total dissolved copper by ICPAES (CSIRO) and GFAAS (DELM). ASV-labile copper results from CSIRO were consistently higher than the ASV-labile copper determined at DELM, possibly due to less loss of copper by adsorption to the Teflon cells at CSIRO.

DOC concentrations in the Harbour were towards the upper end of the normal range for estuaries, with values of 2.2–7 mg/L in the mid-salinity waters. It is well known that dissolved organic matter can bind copper in forms that are less toxic than free metal ion (Teasdale et al 1996). The ability of DOC to bind copper is generally measured as the copper complexing capacity. None of the water samples had significant complexing capacity, in agreement with Teasdale et al (1996). Copper concentrations in all the Macquarie Harbour waters exceeded the complexing capacity of the dissolved organic matter in these waters, indicating that free copper or easily dissociable complexes were present and possibly bioavailable. ASV-labile copper was a significant component of the dissolved copper in all samples (48–65%).

**Table 4.1** Total disssolved metals in Macquarie Harbour waters sampled in October and December, 1995

| Station no | Cu | Mn | Fe | Zn | Cd | Pb | Co | Ni | Al | Ag | Cr |
|---|---|---|---|---|---|---|---|---|---|---|---|
| October sampling | | | | | | | | | | | |
| 3 | 31 | 70 | 62 | 6 | <2 | <10 | 2 | 1 | 131 | <2 | <2 |
| 5 | 33 | 71 | 63 | 6 | <2 | <10 | 6 | 3 | 131 | <2 | <2 |
| 9 | 42 | 99 | 86 | 9 | <2 | <10 | 4 | 3 | 149 | <2 | <2 |
| 11 | 35 | 80 | 73 | 7 | <2 | <10 | <1 | 2 | 135 | <2 | <2 |
| 14 | 26 | 52 | 60 | 5 | <2 | <10 | <1 | 3 | 113 | 2 | <2 |
| 28 | 29 | 62 | 56 | 5 | <2 | <10 | <1 | 2 | 121 | <2 | <2 |
| 32 | 29 | 68 | 72 | 6 | <2 | <10 | 1 | 1 | 137 | <2 | <2 |
| 34 | 26 | 59 | 63 | 5 | <2 | <10 | 2 | <1 | 122 | <2 | <2 |
| December sampling | | | | | | | | | | | |
| 9 | 10 | 17 | 19 | 21 | <2 | 10 | 2 | 2 | 71 | <2 | <2 |
| 14 | 12 | 21 | 23 | 21 | <2 | 10 | 1 | 2 | 76 | <2 | <2 |

**Table 4.2** Copper and dissolved organic carbon in Macquarie Harbour waters sampled in October and December 1995

| Station no | DOC (mg/L) | Total copper (µg/L) | Dissolved copper (µg/L) | | ASV-labile copper (µg/L) | |
|---|---|---|---|---|---|---|
| | | | CSIRO [a] | DELM [b] | CSIRO [a] | DELM [b] |
| October sampling | | | | | | |
| 3 | 6.7 | 46 | 31 | 31 | 20 | 12.2 |
| 5 | 5.2 | 50 | 33 | 36 | 18 | 15.3 |
| 9 | 7.0 | 62 | 42 | 44 | 24 | 16.2 |
| 11 | 6.4 | 50 | 35 | 35 | 19 | 16.7 |
| 14 | 2.8 | 29 | 26 | 26 | 16 | 11.4 |
| 28 | 3.2 | 35 | 29 | 29 | 16 | 10.3 |
| 32 | 4.5 | 61 | 29 | 28 | 14 | 6.5 |
| 34 | 3.0 | 34 | 26 | 23 | 17 | 16.1 |
| December sampling | | | | | | |
| 9 | 2.2 | 12 | 10 | nd[c] | 5.6 | nd |
| 14 | 3.1 | 12 | 12 | nd | 7.3 | nd |

a Analyses by CSIRO, CAAC, Sydney

b Analyses by DELM, Hobart

c not determined

## 4.2 Toxicity of ionic copper and Macquarie Harbour waters to the alga *Nitzschia closterium* (growth inhibition bioassay)

### 4.2.1 Toxicity of ionic copper

Because the salinities of the Macquarie Harbour waters ranged from 15 to 22‰, the growth and toxicity of copper to *Nitzschia closterium* was determined at a range of salinities (table 4.3). Growth rates of the *Nitzschia* controls decreased with decreasing salinity, from 1.2 doublings/day at 25‰ salinity to 0.79 doublings/day at 15‰.

Although growth rates in the controls decreased at lower salinity, there was no significant difference in the toxicity of ionic copper at 15 and 20‰ salinity (tables 4.4 and 4.5 and figs 4.1 and 4.2). The 72-h EC50 for ionic copper was 21 (95% confidence limits of 18–24) and 18 (15–23) µg/L at 15 and 20‰ salinity respectively. Both LOEC and NOEC values were similar at both salinities tested. Copper concentrations as low as 5 µg/L significantly reduced

*Nitzschia* growth rates, indicating that this species should be sensitive enough to detect copper toxicity in the Macquarie Harbour water samples if the copper was bioavailable.

Measured copper concentrations at the beginning and end of the bioassays agreed closely with the nominal copper concentrations, indicating that little copper was lost by adsorption to the flask walls throughout the assay. The pH throughout the bioassays ranged from 7.9–8.3.

**Table 4.3** Toxicity of ionic copper to *Nitzschia closterium* at 20‰ salinity

| Measured copper concentration[a] (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|
| | Divisions/day | Percentage of control | |
| 0 | 0.98 | 100 | 1 |
| 2.5 | 0.83 | 85 | 7 |
| 4.7[b] | 0.73 | 74 | 6 |
| 11[b] | 0.54 | 55 | 15 |
| 22[b] | 0.51 | 52 | 5 |
| 43[b] | 0.50 | 51 | 30 |
| 88[b] | 0.15 | 15 | 41 |

a Copper concentration measured by ICPAES on Day 0 of the bioassay

b The mean cell division rates at these copper concentrations were significantly different to the control mean at $\alpha$ =0.05 by Dunnett's test. This difference corresponds to -21% of the control.

**Table 4.4** Toxicity of ionic copper to *Nitzschia closterium* at 15‰ salinity

| Measured copper concentration[a] (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|
| | Divisions/day | Percentage of control | |
| 0 | 0.79 | 100 | 1 |
| 3 | 0.70 | 89 | 5 |
| 5.6[b] | 0.65 | 82 | 8 |
| 11[b] | 0.58 | 73 | 2 |
| 22[b] | 0.45 | 57 | 0 |
| 43[b] | 0.25 | 32 | 43 |
| 87[b] | 0 | 0 | 47 |

a Copper concentration measured by ICPAES on Day 0 of the bioassay

b The mean cell division rates at these copper concentrations were significantly different to the control mean at $\alpha$ =0.05 by Dunnett's test. This difference corresponds to -16% of the control.

**Table 4.5** Toxicity of ionic copper to *Nitzschia closterium* at varying salinities

| Salinity (‰) | Control cell division rate (doublings/day) | Copper toxicity (µg/L) | | |
|---|---|---|---|---|
| | | 72-h EC50 | LOEC | NOEC |
| 15 | 0.79 | 21 (18–24) | 5.6 | 3 |
| 17.5 | 0.95 | – | – | – |
| 20 | 0.98 | 18 (15–23) | 4.7 | 2.5 |
| 25 | 1.2 | – | – | – |

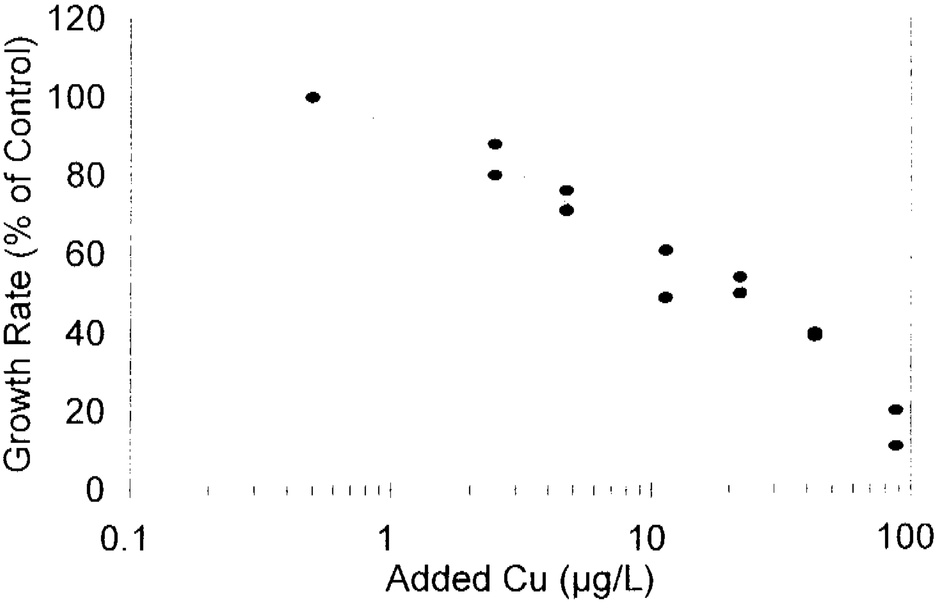



**Figure 4.1** Inhibition of growth of *Nitzschia closterium* by ionic copper at 20‰ salinity

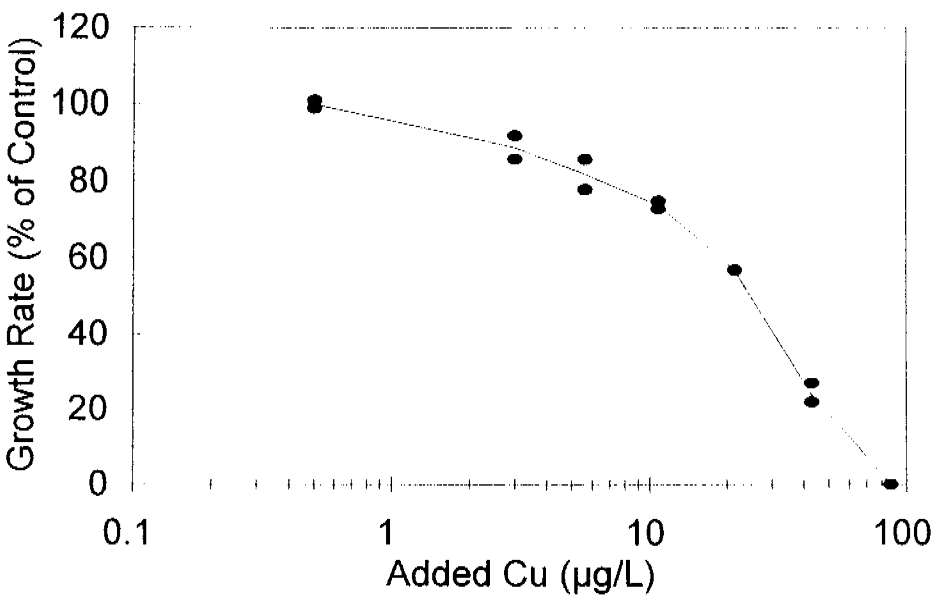



**Figure 4.2** Inhibition of growth of *Nitzschia closterium* by ionic copper at 15‰ salinity

**Table 4.6** Growth of *Nitzschia closterium* in filtered Macquarie Harbour water (station 11)[a] at 15‰ salinity

| Concentration of Macquarie Harbour waters (%) | Mean cell division rate | | CV (%) |
|---|---|---|---|
| | Divisions/day | Percentage of control | |
| 0 | 0.69 | 100 | 8 |
| 6.25 | 0.76 | 110[b] | 7 |
| 12.5 | 0.80 | 116[b] | 1 |
| 25 | 0.88 | 128[b] | 6 |
| 50 | 0.79 | 114[b] | 4 |
| 100 | 0.76 | 110[b] | 2 |

a Station 11 water contained 35µg Cu/L.

b Growth enhancement was not significant at $\alpha = 0.05$ by Dunnett's test.

### 4.2.2 Toxicity of Macquarie Harbour waters (October sampling)

*Filtered waters*

The growth of *Nitzschia closterium* in various dilutions of filtered station 11 water is given in table 4.6. Although full-strength station 11 water (100%) contained 35 µg Cu/L, of which 18 µg Cu/L was ASV-labile, there was no significant decrease in *Nitzschia* growth over the 72-h exposure. Because the *Nitzschia* growth bioassay is sensitive enough to detect copper concentrations as low as 5 µg Cu/L at 15‰ salinity, it appears that <5 µg/L of the copper in the station 11 water was bioavailable. Although algal growth appeared to be enhanced in the Macquarie Harbour water, this was not significant at the alpha=0.05 level by Dunnett's test.

Table 4.7 shows the growth of *Nitzschia* in the other full-strength filtered Macquarie Harbour waters sampled in October, 1995 (all at 18‰ salinity). None of the waters were toxic to *Nitzschia*, with growth rates 88–100% of the controls in clean seawater at 18‰ salinity. Although these waters contained from 26–42 µg Cu/L, less than 5 µg Cu/L was bioavailable otherwise significant decreases in algal growth would have been observed.

**Table 4.7** Growth of *Nitzschia closterium* in filtered Macquarie Harbour waters at 18‰ salinity

| Station no | Dissolved Cu (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|---|
| | | Divisions/day | Percentage of control | |
| Control | | 1.0 | 100 | 3 |
| 3 | 31 | 0.88 | 88 | 16 |
| 5 | 33 | 0.98 | 98 | 7 |
| 9 | 42 | 1.0 | 100 | 3 |
| 14 | 26 | 1.0 | 100 | 2 |
| 28 | 29 | 1.1 | 110 | 6 |
| 32 | 29 | 1.0 | 100 | 4 |
| 34 | 26 | 1.0 | 100 | 4 |

*Unfiltered waters*

To determine whether toxicity of the Macquarie Harbour waters may be associated with particulate material, the toxicity of six of the unfiltered waters from the October sampling was investigated (table 4.8). Two concentrations of each sample (100% and 50%, diluted with 18‰ salinity clean seawater) were tested in triplicate. There was no significant decrease in algal growth in any of the water samples tested, despite total copper concentrations ranging from 29–62 µg Cu/L. No significant growth enhancement was observed.

### 4.2.3 Toxicity of Macquarie Harbour waters (December sampling)

Growth of *Nitzschia* in filtered and unfiltered Macquarie Harbour water collected from station 9 in December 1995 (21‰ salinity) is shown in table 4.9. A small but significant decrease in algal growth rate in full-strength (100%) filtered and unfiltered water was observed, with rates 88% and 81% of the controls respectively. However, this small decrease in algal growth was far less than that predicted by ASV-labile copper determinations. There was no effect on algal growth at concentrations of station 9 water of 75% or less ie the NOEC was 75%.

Similar results were found for filtered and unfiltered station 14 water (table 4.10). A small but significant decrease in algal growth rate in full-strength (100%) filtered and 100% and 75% unfiltered water was observed, with rates 88%, 92% and 92% of the controls respectively. The LOEC for the filtered water was 100%, with an NOEC of 75%. The unfiltered water showed similar toxicity with an LOEC of 75% and an NOEC of 50%.

Copper concentrations in each dilution of filtered and unfiltered Macquarie Harbour water were measured on Day 0 and Day 3 of the bioassays (table 4.11). There was excellent agreement between nominal and measured copper concentrations at each dilution. Copper concentrations at the end of the bioassay were the same as at the beginning of the bioassay confirming that copper did not adsorb to the walls of the silanised bioassay flasks. Thus the lack of toxicity of the Macquarie Harbour water samples was not due to losses of copper to the test containers.

**Table 4.8** Growth of *Nitzschia closterium* in unfiltered Macquarie Harbour waters at 18‰ salinity

| Station no | Concentration (%) | Mean cell division rate | | CV (%) |
|---|---|---|---|---|
| | | Divisions/day | Percentage of control | |
| Control | | 1.0 | 100 | 2 |
| 3 | 100[a] | 1.2 | 120 | 22 |
| | 50[b] | 1.0 | 100 | 2 |
| 5 | 100 | 0.94 | 94 | 3 |
| | 50 | 1.0 | 100 | 3 |
| 9 | 100 | 1.0 | 100 | 3 |
| | 50 | 1.1 | 110 | 3 |
| 11 | 100 | 1.1 | 110 | 18 |
| | 50 | 1.1 | 110 | 6 |
| 14 | 100 | 1.0 | 100 | 2 |
| | 50 | 1.0 | 100 | 4 |
| 28 | 100 | 1.1 | 110 | 6 |
| | 50 | 1.1 | 110 | 8 |

a 100% is full strength Macquarie Harbour water

b 50% is full strength (100%) Macquarie Harbour water diluted 1:2 with 18‰ salinity seawater

**Table 4.9** Growth of *Nitzschia closterium* in filtered and unfiltered Macquarie Harbour water, station 9 (21‰, December 1995 sampling)

| Concentration of station 9 water (%) | Measured copper concentration (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|---|
| | | Divisions/day | Percentage of control | |
| Unfiltered | | | | |
| 0 | -[b] | 1.2 | 100 | 4 |
| 50 | - | 1.2 | 100 | 6 |
| 75 | - | 1.1 | 92 | 5 |
| 100[a] | - | 0.97 | 81 | 7 |
| Filtered | | | | |
| 0 | <1 | 1.1 | 100 | 2 |
| 12.5 | 2 | 1.1 | 100 | 1 |
| 25 | 3 | 1.1 | 100 | 2 |
| 50 | 6 | 1.0 | 91 | 2 |
| 75 | 7 | 1.1 | 100 | 7 |
| 100[a] | 10 | 0.97 | 88 | 2 |

a the mean cell division rates at full strength Macquarie Harbour water were significantly lower than the controls at $\alpha = 0.05$

b not determined

**Table 4.10** Growth of *Nitzschia closterium* in filtered and unfiltered Macquarie Harbour water station 14 (20°/oo, December 1995 sampling)

| Concentration of station 14 water (%) | Measured copper concentration (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|---|
| | | Divisions/day | Percentage of control | |
| Unfiltered | | | | |
| 0 | -[b] | 1.2 | 100 | 4 |
| 50 | - | 1.2 | 100 | 6 |
| 75[a] | - | 1.1 | 92 | 3 |
| 100[a] | - | 1.1 | 92 | 4 |
| Filtered | | | | |
| 0 | <1 | 1.1 | 100 | 4 |
| 12.5 | 2 | 1.1 | 100 | 3 |
| 25 | 3 | 1.1 | 100 | 6 |
| 50 | 5 | 1.1 | 100 | 2 |
| 75 | 9 | 1.2 | 109 | 1 |
| 100[a] | 11 | 0.96 | 88 | 3 |

a the mean cell division rates for these concentrations of Macquarie Harbour waters were significantly lower than the controls at $\alpha$ = 0.05 by Dunnett's test

b not determined

**Table 4.11** Nominal and measured copper concentrations in Macquarie Harbour waters (stations 9 and 14, December 1995 sampling) at the beginning and end of the *Nitzschia closterium* bioassay

| Sample station | Concentration (%) | Nominal copper concentration (µg/L) | Measured copper concentration (µg/L) | |
|---|---|---|---|---|
| | | | Day 0 | Day 3 |
| 9 | 100 | 10 | 10 | 10 |
| | 75 | 7.5 | 7 | 7 |
| | 50 | 5 | 6 | 5 |
| | 25 | 2.5 | 3 | 3 |
| | 12.5 | 1.3 | 2 | 3 |
| 14 | 100 | 12 | 11 | 10 |
| | 75 | 9 | 9 | 8 |
| | 50 | 6 | 5 | 6 |
| | 25 | 3 | 3 | 4 |
| | 12.5 | 1.5 | 2 | 2 |

### 4.2.4 Copper complexing capacity of Macquarie Harbour waters

The copper complexing capacity of filtered station 9 and unfiltered station 14 water (collected in December, 1995) was determined by spiking full-strength water with various amounts of ionic copper (5–80 µg/L) and measuring the decrease in algal growth rate over 72 h.

Copper additions as high as 20 µg/L (total dissolved copper=32 µg/L) to the filtered station 9 water had no effect on algal growth (table 4.12). The first observable effect on algal growth rate was at 52 µg Cu/L, an order of magnitude higher than copper concentrations required to first affect algal growth in clean seawater at the same salinity. The 72-h EC50 was approximately 90 µg Cu/L, with broad 95% confidence limits of 60–138 µg Cu/L. This is also much higher than the 72-h EC50 of 18 µg Cu/L in clean seawater at 20‰ salinity. The 72-h EC15 value (a measure of the copper complexing capacity of the water) was approximately 20 µg/L. Although the station 9 water contained 10 µg Cu/L initially, of which 5.6 µg Cu/L was ASV-labile, further copper additions to this water were complexed or bound and not available for uptake into the algal cells to cause toxicity until copper concentrations exceeded 20 µg/L.

**Table 4.12** Growth of *Nitzschia closterium* in copper-spiked, filtered Macquarie Harbour water, station 9 (21‰ salinity)

| Added copper (µg/L) | Total copper[b] (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|---|
| | | Divisions/day | % control | |
| 0 | 10 | 0.92 | 100 | 11 |
| 5 | 15 | 0.86 | 93 | 7 |
| 10 | 21 | 0.93 | 101 | 2 |
| 20 | 32 | 0.89 | 97 | 9 |
| 40[a] | 52 | ᴗ.70 | 76 | 8 |
| 80[a] | 95 | 0.47 | 51 | 4 |

a The mean cell division rates at these added copper concentrations were significantly less than the control mean at a = 0.05 by Dunnett's test. This corresponds to -14% at the control.

b Total copper (copper in station 9 water plus copper added) was measured by ICPAES on Day 0.

Similar results were found for unfiltered station 14 water spiked with ionic copper (table 4.13). Total dissolved copper concentrations up to 22 µg/L had no effect on algal growth rate, whereas copper concentrations exceeding 42 µg/L significantly decreased algal growth. The 72-h EC50 was 117 µg Cu/L (95% confidence limits of 100–144 µg/L) and the copper complexing capacity of the sample (EC15) was 42 µg/L (34–50 µg /L). Again, further copper additions to this water were not bioavailable until copper concentrations exceeded the complexing capacity of the sample (42 µg Cu/L).

**Table 4.13** Growth of *Nitzschia closterium* in copper-spiked, unfiltered Macquarie Harbour water station 14 (21‰ salinity, December 1995 sampling)

| Added copper (µg/L) | Total copper[b] (µg/L) | Mean cell division rate | | CV (%) |
|---|---|---|---|---|
| | | Divisions/day | % control | |
| 0 | 12 | 0.84 | 100 | 6 |
| 10 | 22 | 0.83 | 99 | 2 |
| 30[a] | 42 | 0.75 | 89 | 5 |
| 60a | 72 | 0.68 | 81 | 5 |
| 90[a] | 102 | 0.52 | 62 | 2 |
| 120[a] | 132 | 0.37 | 44 | 14 |

a The mean cell division rates for these added copper concentrations were significantly less than the control mean at $\alpha = 0.05$ by Dunnett's test. This corresponds to -9% of the control.

b Total copper (copper in station 14 water plus copper added) was measured by ICPAES on Day 0.

These results were surprising because chemical complexing capacity experiments using ASV had shown that these waters had no copper complexing capacity and indeed contained 'free' copper. This ASV-labile copper represents ionic copper, inorganic copper complexes and organic copper complexes which can dissociate at the ASV electrode. It is generally (and simplistically) assumed that this ASV-labile copper is similar to the bioavailable fraction. However, the *Nitzschia* growth inhibition bioassays showed that none of this copper was able to penetrate the algal cell membrane where it could exert a toxic effect on cell division. Moreover, additional copper could be added to the waters and a decrease in algal growth rate was not observed until copper concentrations exceeded 20 and 42 µg Cu/L in filtered station 9 and unfiltered station 14 waters respectively. This apparent discrepancy between chemically labile and bioavailable copper was investigated further in a series of additional experiments outlined below.

#### 4.2.5 Prevention of copper toxicity to *Nitzschia* growth: Inorganic versus organic binding

Once dissolved copper in the Macquarie Harbour waters exceeds the complexing capacity of the dissolved organic matter (such as humic and fulvic acids), the free copper would be detected as ASV-labile copper and potentially bioavailable. The lack of toxicity of the water samples to algae suggests that this ASV-labile copper is not bioavailable, at least to algae. It is possible that this copper is present as organic complexes which are directly reduced at the ASV electrode (rather than first dissociating to free copper which then diffuses to the mercury electrode and is reduced) so it is detected as ASV-labile copper, whereas the organic copper complex itself does not dissociate at the algal cell membrane and therefore cannot penetrate the cell to cause a toxic effect on algal growth. If this were the case, UV irradiation of the water sample should break down the organic copper complexes so that more copper is available to inhibit algal growth.

In two experiments, filtered Macquarie Harbour water from station 9 (October sampling) containing 42 µg Cu/L and station 14 (December sampling) containing 12 µg Cu/L, were irradiated under UV light for 4 h. The toxicity of the UV irradiated water was then compared with the toxicity of the non-irradiated water over 72 h in the *Nitzschia* growth inhibition test. In both waters the toxicity of the irradiated and non-irradiated water was the same. This suggests that either copper was still bound in a non-bioavailable form or that copper losses during UV irradiation occurred. It appears unlikely that copper was solely bound as organic complexes otherwise a reduction in algal growth after copper release from the organic complexes would have been detected.

It is possible that copper in the Macquarie Harbour waters is adsorbed to humic coated colloidal inorganic compounds, such as manganese, iron or aluminium oxyhydroxides (Teasdale et al 1996). A portion of these colloids may dissociate at the ASV electrode (and are therefore measured as ASV-labile copper) and adsorb to, but do not penetrate, the algal cell membrane. Previous work in our laboratory has shown that copper adsorbs to manganese, iron, cobalt and aluminium hydroxides on the *Nitzschia* cell surface and prevents copper from penetrating into the cell to cause an inhibitory effect on cell division (Stauber & Florence 1985a, b, 1987). Concentrations of manganese, cobalt, aluminium and iron as low as 4, 2, 110 and 550 µg/L respectively could reduce the toxic effect of copper by 2.5 to 5 times. Copper concentrations of over 50 µg/L were required to reduce algal growth rates by 50%. Manganese and cobalt were the most effective in reducing copper toxicity because they could also catalytically scavenge free radicals.

Concentrations of dissolved manganese (17–99 µg/L) and aluminium (71–149 µg/L) in the Macquarie Harbour waters, if present as colloidal oxides/hydroxides, were well in excess of the concentrations required to protect *Nitzschia* from copper toxicity. Iron (19–86 µg/L) and cobalt (<1–6 µg/L) in these waters may also protect to a lesser extent. To test this hypothesis, we added 20 µg Mn/L (as manganese chloride) and/or 20 µg Fe/L (as iron(III) chloride) to clean seawater at 20‰ salinity containing *Nitzschia* cells and allowed the flasks to stand for three days, prior to the addition of copper (10–100 µg/L). Bioassays were then run as usual and the inhibitory effect of copper in the presence of manganese or iron compared with alone. There were no differences in the toxicity of copper in the presence or absence of iron and manganese. It is possible that three days was not long enough for oxidation of the added Mn(II) and Fe (III) at the cell surface, to form hydroxides and prevent copper penetration into the cell. It is also possible that colloidal aluminium, iron (including humic coated iron oxide), manganese and cobalt are together contributing to prevent copper uptake by this or another as yet unknown mechanism.

Removal of colloidal manganese and/or adsorbed copper from the Macquarie Harbour waters (station 9 and 14) was also attempted by ultrafiltration through an Amicon Centricon 10 (10,000 molecular weight cut-off). It was hoped that by determining the metal concentrations and toxicity of the waters before and after removal of this fraction, it may help indicate to what the copper was bound. However, all the manganese and 62–100% of the copper passed through the membrane indicating that most of these metals were present in dissolved forms with less than 10,000 molecular weight. Further work is required to understand the speciation of copper in Macquarie Harbour waters and its impact on algae.

## 4.3 Toxicity of ionic copper and Macquarie Harbour waters to the alga *Dunaliella tertiolecta* (enzyme inhibition bioassay)

### 4.3.1 Toxicity of ionic copper

The effect of ionic copper on the activity of the enzyme ß-D-galactosidase in the marine green alga *Dunaliella tertiolecta* at 17‰ salinity is shown in table 4.14 and fig 4.3. This bioassay was extremely sensitive to copper, with concentrations of copper as low as 2.5 µg/L significantly decreasing algal enzyme activity (86% of controls). The 72-h EC50 was 18 µg Cu/L, with 95% confidence limits of 15–22 µg/L. Thus this enzyme inhibition bioassay showed similar sensitivity to the *Nitzschia* growth inhibition test, and should be able to detect toxicity in the Macquarie Harbour waters if the copper is present in a bioavailable form.

### 4.3.2 Toxicity of Macquarie Harbour waters

Filtered Macquarie Harbour waters from the October and December samplings were assayed for toxicity using the *Dunaliella* enzyme inhibition bioassay. Due to the bioassay procedure, 90% was the maximum concentration of sample that could be assayed. Controls were matched to the same salinity as each of the water samples.

Inhibition of ß-D-galactosidase activity in *Dunaliella* in each of the water samples is given in table 4.15. Significant inhibition of enzyme activity (46–72%) was observed in all the Macquarie Harbour water samples tested, with station 5 being the most toxic. Enzyme inhibition was weakly correlated with dissolved copper concentrations in the samples ($r^2$=0.65).

**Table 4.14** Effect of ionic copper on β-galactosidase activity in *Dunaliella tertiolecta* at 17‰ salinity

| Added copper (µg/L) | β-Galactosidase activity (% of control) | CV (%) |
|---|---|---|
| 0 | 100 | 4 |
| 2.5[a] | 86 | 3 |
| 5[a] | 78 | 3 |
| 10[a] | 60 | 5 |
| 20[a] | 46 | 4 |
| 40[a] | 35 | 9 |

a β-galactosidase activity at these copper concentrations was significantly lower than the control at $\alpha$ = 0.05 by Dunnett's test. This corresponds to -10% of the control.

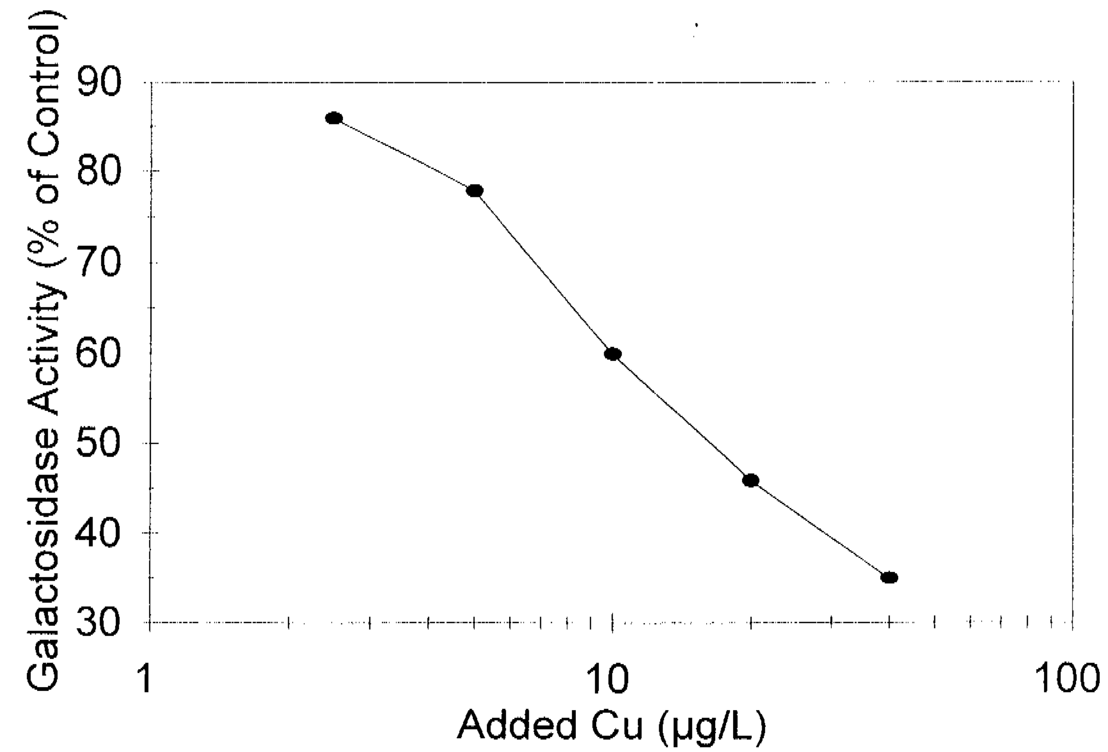



**Figure 4.3** The effect of ionic copper on the activity of β-D-galactosidase in *Dunaliella tertiolecta* at 17‰ salinity

Because the enzyme bioassay is carried out 44.5°C, it was important to determine if toxicity of the samples was likely at realistic water temperatures or was due simply to breakdown of the copper complexes at this high temperature. A portion of the station 14 water was autoclaved at 121°C for 20 min, and the toxicity of the autoclaved and non-autoclaved water to ß-D-galactosidase activity in *Dunaliella* assessed using the standard enzyme bioassay. It was assumed that if high temperatures caused dissociation of the copper complexes, more copper would be bioavailable leading to greater enzyme inhibition in the assay with the autoclaved sample. Enzyme inhibition in both the autoclaved and non-autoclaved sample was the same (82% of the control). It appears that copper complexes present in the sample do not dissociate at high temperatures and suggests that copper bioavailability and toxicity in these samples is not influenced by the incubation temperature of 44.5°C in the enzyme inhibition bioassay.

Using the ß-D-galactosidase inhibition versus ionic copper calibration graph, the inhibition of ß-D-galactosidase activity in the Macquarie Harbour water samples was converted to an estimate of bioavailable copper. Bioavailable copper (determined in this way) and ASV-labile copper (determined electrochemically) for each of the Macquarie Harbour waters are shown in table 4.16 and fig 4.4. There was a weak correlation between the two measurements ($r^2$= 0.51).

Though there was reasonable agreement between ASV-labile copper and bioavailable copper estimated from the galactosidase inhibition bioassay, this was not the case for bioavailable copper determined in the growth inhibition test. It is possible that the enzyme ß-D-galactosidase is located on the cell membrane and that copper, adsorbed to iron, manganese and aluminium hydroxides at the cell surface, can inhibit the enzyme on the cell membrane. Alternatively, the enzyme may compete with carrier proteins (which transport copper into the cell) leading to copper inhibition of the enzyme at the cell surface. However, because copper is bound to the metal hydroxides at the cell surface, less copper penetrates the cell and therefore an effect on cell division is not observed.

**Table 4.15** Inhibition of β-galactosidase activity in *Dunaliella tertiolecta* in filtered Macquarie Harbour waters (90%)[a]

| Station no | Salinity (‰) | Dissolved copper concentration (µg/L) | β-galactosidase activity (% of control) | CV (%) |
|---|---|---|---|---|
| October Sampling | | | | |
| 3 | 17 | 31 | 55 | 4 |
| 5 | 17 | 33 | 46 | 1 |
| 9 | 12 | 42 | 60 | 1 |
| 11 | 14 | 35 | 55 | 3 |
| 14 | 17 | 26 | 62 | 2 |
| 28 | 17 | 29 | 50 | 2 |
| 32 | 17 | 28 | 63 | 2 |
| 34 | 17 | 23 | 56 | 2 |
| December Sampling | | | | |
| 9 | 21 | 10 | 72 | 3 |
| 14 | 20 | 12 | 66 | 6 |

a Full strength (100%) Macquarie Harbour waters could not be tested, due to additional reagents added in the enzyme assay

## 4.4 Toxicity of ionic copper and Macquarie Harbour waters to the amphipod *Allorchestes compressa*

### 4.4.1 Toxicity of ionic copper

The effect of ionic copper (3–16 µg/L) on the growth and survival of *Allorchestes compressa* over 27 days is shown in table 4.17. Throughout the experiment, the temperature ranged from 18 to 19°C, pH from 8.0 to 8.3, salinity from 21 to 23‰ and dissolved oxygen from 5.3 to 6.9 mg/L. Measured copper concentrations in each tank were relatively constant over the 27-day experiment, but were generally lower than nominal copper concentrations, possibly due to absorption of copper onto the glass tank walls. Results from the bioassays are therefore expressed in terms of measured copper concentrations.

Survival in the controls over the 27 days ranged from 67 to 87% (mean 77%), and was lower than the 90–100% expected. Similar survival (53–87%) was found at all copper concentrations tested. Only the mean amphipod survival at 4 µg Cu/L was significantly lower than the controls at alpha = 0.05 by Dunnett's test.

Amphipod growth was a more sensitive and reliable endpoint for copper toxicity than amphipod survival. Growth of the amphipods over 27 days was significantly lower than the controls at copper concentrations of 4–16 µg/L. The LOEC was 4 µg Cu/L and the NOEC was 3 µg Cu/L. The 27-day EC50 was 15 µg Cu/L, with 95% confidence limits of 13-17 µg Cu/L, using trimmed Spearman Karber analysis. Because dissolved copper concentrations in the Macquarie Harbour waters ranged from 10 to 42 µg Cu/L, the 27-day amphipod growth bioassay should be sensitive enough to detect copper toxicity in these waters if the copper is present in a bioavailable form.

### 4.4.2 Toxicity of Macquarie Harbour waters

The growth and survival of *Allorchestes compressa* in Macquarie Harbour station 9 water over 27 days is shown in table 4.18. Throughout the experiment, the temperature ranged from 18 to 19°C, the pH ranged from 7.8 to 8.2, salinity from 21 to 22‰ and dissolved oxygen from 5.3 to 6.9 mg/L. The measured copper concentrations throughout the bioassay agreed well with the expected copper concentrations in the water samples, except for 100% station 9 water which contained 6 µg Cu/L instead of 10 µg Cu/L.

**Table 4.16** Bioavailable copper (from the β-galactosidase inhibition bioassay) compared with ASV-labile copper in Macquarie Harbour waters

| Station no | Date sampled | Bioavailable copper[a] (µg/L) | ASV-labile copper[b] (µg/L) |
|---|---|---|---|
| 3 | Oct 1995 | 13.5 | 20 |
| 5 | Oct 1995 | 20 | 18 |
| 9 | Oct 1995 | 10 | 24 |
| 9 | Dec 1995 | 6.5 | 5.6 |
| 11 | Oct 1995 | 13.5 | 19 |
| 14 | Oct 1995 | 9.5 | 16 |
| 14 | Dec 1995 | 8 | 7.3 |
| 28 | Oct 1995 | 17 | 16 |
| 32 | Oct 1995 | 9 | 14 |
| 34 | Oct 1995 | 12 | 17 |

a Bioavailable copper was determined from the inhibition of β-galactosidase activity in *D. tertiolecta* (as a % of the control) using the ionic copper calibration graph

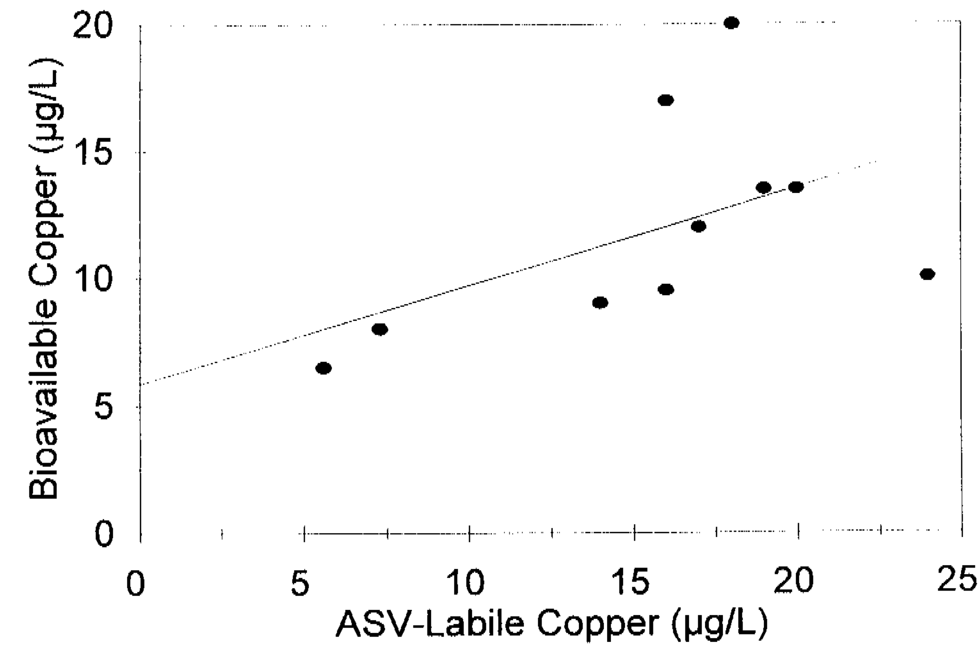



**Figure 4.4** Bioavailable copper (determined from β-D-galactosidase bioassay) versus ASV-labile copper (determined electrochemically)

**Table 4.17** Toxicity of ionic copper to *Allorchestes compressa* after a 27-day exposure

| Measured copper concentration (µg/L) | Mean amphipod survival | | Amphipod growth | |
|---|---|---|---|---|
| | (% recovery) | CV (%) | Mass per amphipod (mg) | CV (%) |
| 0 | 77 | 11 | 0.24 | 9 |
| 3 | 87 | 7 | 0.25 | 8 |
| 4 | 53[a] | 40 | 0.16[b] | 26 |
| 8 | 83 | 7 | 0.18[b] | 10 |
| 13 | 76 | 7 | 0.14[b] | 14 |
| 16 | 78 | 9 | 0.11[b] | 8 |

a The mean % recovery at this copper concentration was significantly lower than the control at $\alpha = 0.05$ by Dunnett's test.

b The mean mass per amphipod was significantly lower than the control at $\alpha = 0.05$ by Dunnett's test.

**Table 4.18** Growth and survival of *Allorchestes compressa* in Macquarie Harbour, station 9 water

| Concentration of station 9 water (%) | Measured Cu concentration (µg/L) | Mean amphipod survival | | Amphipod growth | |
|---|---|---|---|---|---|
| | | % recovery | CV (%) | Mean mass per amphipod (mg) | CV (%) |
| Control | 1 | 104 | 3 | 0.28 | 8 |
| 12.5 | 2 | 98 | 3 | 0.26 | 9 |
| 25 | 2 | 99 | 4 | 0.25 | 5 |
| 50 | 4 | 101 | 3 | 0.26 | 11 |
| 100 | 6 | 100 | 7 | 0.27 | 5 |

Mean amphipod survival (% recovery) in the controls was 104%, possibly because more than 30 amphipods were added initially. Amphipod survival ranged from 98 to 104% and was not reduced at any concentration of station 9 water tested. Similarly, amphipod growth (0.25–0.28 mg per amphipod) was not affected at any concentration tested. The highest measured concentration of copper in the station 9 water (100%) was 6 µg Cu/L and it appears that <4 µg/L of this copper was available to cause toxicity to the amphipod.

The growth and survival of *Allorchestes compressa* in Macquarie Harbour station 14 water over 27 days is shown in table 4.19. Throughout the experiment, the temperature was maintained at 18°C, the pH ranged from 7.9 to 8.2, salinity from 19 to 22‰ and dissolved oxygen from 4.9 to 6.9 mg/L.

Concentrations of station 14 water (25–100%) containing 5–10 µg Cu/L, had no effect on amphipod survival, which ranged from 86-99%. There was a small but statistically significant effect on amphipod survival at the lowest concentration tested (12.5%). This result is unlikely to be biologically meaningful, because higher concentrations of station 14 water had no effect.

Growth of the amphipods over 27 days was significantly reduced in all concentrations of station 14 water. The LOEC was 12.5%, corresponding to a copper concentration of 3 µg Cu/L. Within the error of the copper determination by ICPAES (3 ± 1 µg/L), this is similar to the LOEC of 4 µg Cu/L for ionic copper alone. It appears that some of the copper in the station 14 water was bioavailable, and that this contributed to the toxicity of the station 14 water to amphipod growth.

In summary, the Macquarie Harbour waters had no effect on amphipod survival as predicted from the ionic copper experiments. However, station 14 water did reduce amphipod growth and this growth inhibition was possibly due to copper in the sample. No effect of station 9 water on amphipod growth was observed.

Ahsanullah and Williams (1991) investigated the sub-lethal effects of copper (and zinc, cadmium and chromium) on survival, average weight and biomass (average weight x survival proportion) of *Allorchestes compressa* at 19°C and 31‰ salinity. Copper concentrations producing minimum detectable decreases in mean survival, weight and biomass (equivalent to LOEC values) were 24, 5.2 and 3.7 µg Cu/L respectively. The LOEC for mean weight (5.2 µg Cu/L) reported by Ahsanullah and Williams (1991) was similar to the LOEC found in the present study (4 µg Cu/L) despite the different salinities used.

**Table 4.19** Growth and survival of *Allorchestes compressa* in Macquarie Harbour, station 14 water

| Concentration of station 14 water (%) | Measured Cu concentration (µg/L) | Mean amphipod survival | | Amphipod growth | |
|---|---|---|---|---|---|
| | | % recovery | CV (%) | Mean mass per amphipod (mg) | CV (%) |
| Control | 2 | 99 | 7 | 0.32 | 11 |
| 12.5 | 3 | 82[a] | 18 | 0.23[b] | 22 |
| 25 | 5 | 95 | 7 | 0.24[b] | 12 |
| 50 | 6 | 90 | 3 | 0.20[b] | 9 |
| 100 | 10 | 86 | 12 | 0.20[b] | 4 |

a The mean survival at this concentration was significantly less than the control at $\alpha = 0.05$ by Dunnett's test.

b The mean amphipod mass at these concentrations was significantly less than the control at $\alpha = 0.05$ by Dunnett's test.

## 4.5 Toxicity of ionic copper and Macquarie Harbour waters to juvenile flounder *Rhombosolea tapirina*

### 4.5.1 Survival

Survival of juvenile flounder after a 14-day exposure to ionic copper and Macquarie Harbour water (station 9 and 14) is shown in table 4.20. In general, measured copper concentrations were close to nominal concentrations throughout the 14-day bioassay.

There was no significant effect on survival in any of the Macquarie Harbour waters tested (at alpha = 0.05 by Dunnett's test). Copper concentrations of 0–80 µg/L also had no significant effect on flounder survival, with a NOEC at 80 µg Cu/L. Survival of flounder was only reduced at the highest copper concentration tested (160 µg/L). Most mortalities occurred within the first 24 hours of exposure and the last fish died on day 8 of the test.

The 14-day EC50 was 138 µg Cu/L, with 95% confidence limits of 126–152 µg Cu/L (based on nominal concentrations). This was well above expected concentrations of dissolved copper in mid-depth Macquarie Harbour waters at 21‰ salinity. Flounder survival, like amphipod survival, was not a sensitive test endpoint for copper.

Juvenile flounder were more sensitive to copper at 20‰ salinity over 14 days than juvenile glass perch and juvenile scaled mullet in 4-day tests at 20‰ salinity (Denton & Burdon-Jones 1986). They reported 96-h LC50 values of 6 and 2.65 mg Cu/L for glass perch and mullet respectively.

### 4.5.2 Copper content of fish

Residues of copper in juvenile flounder after a 14-day exposure to ionic copper were significantly higher at the 0.05 level than control fish at all copper concentrations tested (table 4.21), with the highest copper content in fish exposed to 160 µg Cu/L. There was high variability in the copper contents and in some treatments there were significant differences between containers, particularly in the treatments which resulted in greater accumulation of copper in the fish.

There was no significant difference (at the 0.05 level) in the copper content of fish exposed to Macquarie Harbour water (both concentrations of station 9 and 14 water) compared with controls. Even though dissolved copper concentrations in these waters were similar to the dissolved copper concentrations in the ionic copper treatments (7 and 11 µg Cu/L) in which there were significant increases, there was no significant accumulation of copper from Macquarie Harbour waters. This suggests that the copper present in the Macquarie Harbour waters was not available for uptake into fish tissue over 14 days.

**Table 4.20** Survival of juvenile flounder after a 14-day exposure to ionic copper or Macquarie Harbour waters (stations 9 and 14)

| Treatment | Measured copper concentration (μg/L) | Mean survival (%) | CV (%) |
|---|---|---|---|
| Control | 2 | 100 | 0 |
| 5 μg Cu/L | 7 | 97 | 7 |
| 10 μg Cu/L | 11 | 98 | 4 |
| 20 μg Cu/L | 23 | 97 | 4 |
| 40 μg Cu/L | 52 | 100 | 0 |
| 80 μg Cu/L | 76 | 96 | 4 |
| 160 μg Cu/L[a] | 170 | 38 | 32 |
| 100% station 9 | 13 | 100 | 0 |
| 50% station 9 | 7 | 100 | 0 |
| 100% station 14 | 13 | 100 | 0 |
| 50% station 14 | 6 | 100 | 0 |

a The mean survival at this copper concentration was significantly less than the control at $\alpha = 0.05$ by Dunnett's test.

The accumulation of copper in the body of the fish was relatively low. This may be due to the short exposure time, as it was shown that there were no significant differences in whole body copper levels in rainbow trout (*Oncorhynchus mykiss*) exposed to 0–194 μg Cu/L after 14 days exposure (Dixon & Sprague 1981). These authors, however, did report significant increases in copper accumulation after 21 days.

### 4.5.3 Histopathological changes

Table 4.22 shows the prevalence of the most common histopathological changes in juvenile flounder after a 14-day exposure to copper and Macquarie Harbour waters. The main organs affected by exposure to copper were gills and liver, in agreement with Baker (1969). The flounder which were exposed to copper showed epithelial necrosis (death of individual cells), epithelial lifting (lifting of respiratory epithelium covering the lamellae, which increases the respiratory diffusion distance and thus can affect physiological processes such as gas exchange), and proliferation of chloride cells in their gills (fig 4.5). The prevalence and degree of these changes seemed to be related to the concentration to which the fish were exposed and generally increased with increasing copper concentration (with the exception of the fish exposed to 5 μg Cu/L).

**Table 4.21** Residues of copper in juvenile flounder after a 14-day exposure to ionic copper or Macquarie Harbour water (stations 9 and 14)

| Treatment | Measured copper concentration (μg/L) | Mean copper content (mg/kg dry weight) | CV (%) |
|---|---|---|---|
| Control | 2 | 2.01 | 13 |
| 5 μg Cu/L[a] | 7 | 3.99 | 23 |
| 10 μg Cu/L[a] | 11 | 2.89 | 14 |
| 20 μg Cu/L[a] | 23 | 3.08 | 13 |
| 40 μg Cu/L[a] | 52 | 4.46 | 38 |
| 80 μg Cu/L[a] | 76 | 3.92 | 21 |
| 160 μg Cu/L[a] | 170 | 11.55 | 79 |
| 100% station 9 | 13 | 1.97 | 29 |
| 50% station 9 | 7 | 2.31 | 19 |
| 100% station 14 | 13 | 2.60 | 36 |
| 50% station 14 | 6 | 2.28 | 18 |

a Copper contents of flounder at these concentrations were significantly greater than the control at $\alpha = 0.05$ by Dunnett's test (after transformation)

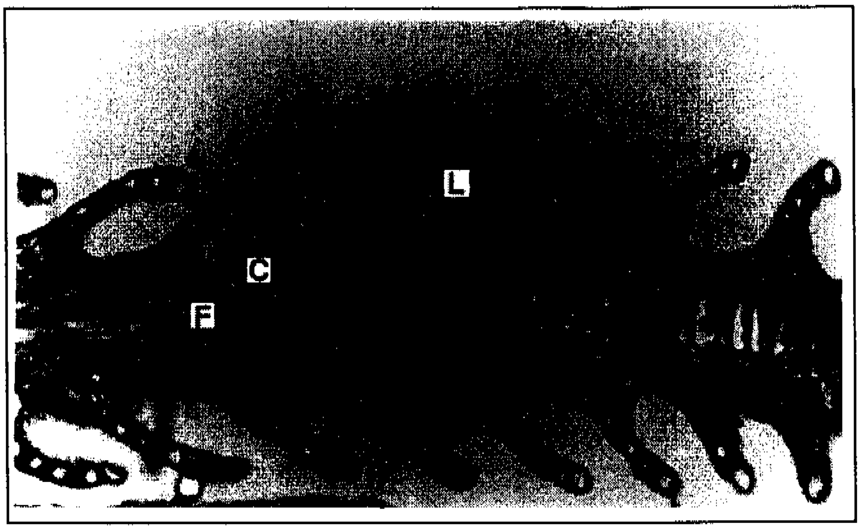
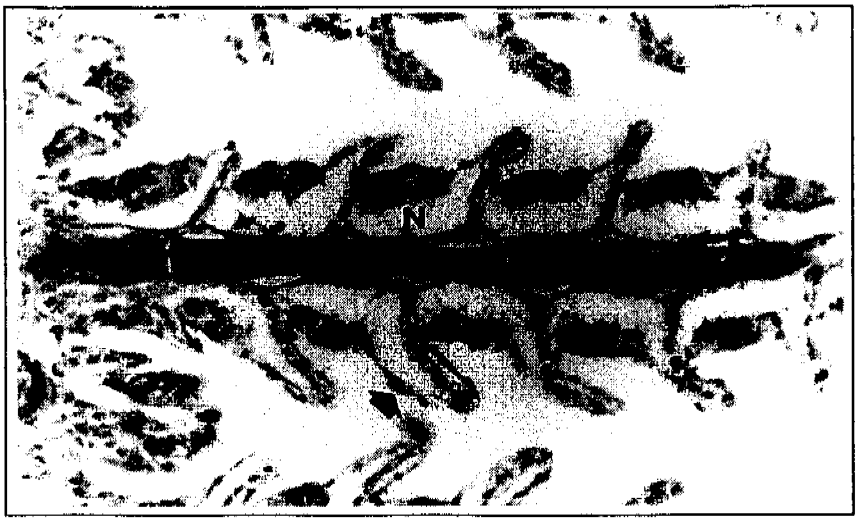



**Figure 4.5a** Gill filament from a control fish (F – filament, L – lamellae, C – chloride cell)



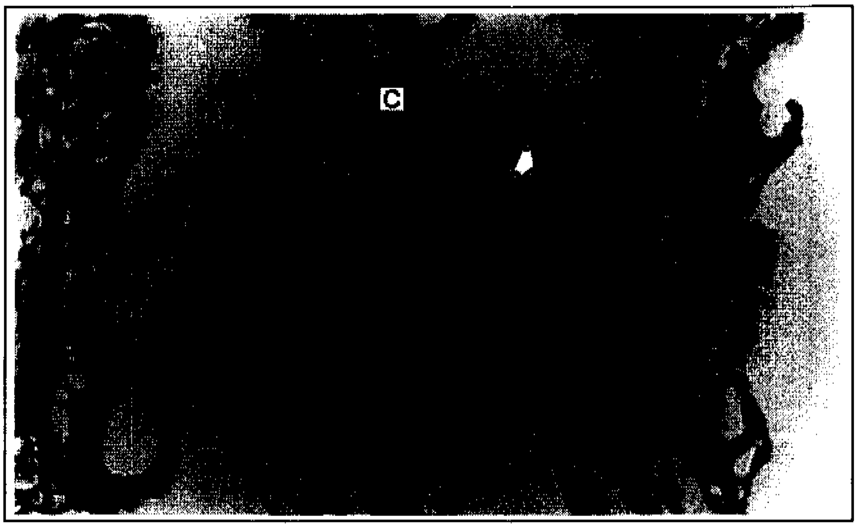




**Figure 4.5b, c** Gill filament from an exposed fish (N: necrosis, arrow: lifted epithelium, open arrow: lamellae fusion)

*Epithelial lifting and necrosis*

Significant epithelial lifting and necrosis occurred at copper concentrations of 40–160 μg/L, with an LOEC of 40 μg Cu/L and no effect (NOEC) at 20 μg Cu/L. The 14-day EC50 for epithelial lifting was 22 μg Cu/L, with 95% confidence limits of 19–26 μg Cu/L. Epithelial necrosis was slightly less sensitive as an endpoint with a 14-day EC50 of 35 μg Cu/L (30–39 μg Cu/L). Although dissolved copper concentrations in the Macquarie Harbour waters were lower than the copper concentrations which would cause a significant effect, significant epithelial lifting and necrosis was observed at all concentrations of Macquarie Harbour water tested (both station 9 and 14). This suggests that copper was not solely responsible for the toxic effects of Macquarie Harbour water and that some other metal, combination of metals or other compounds in Macquarie Harbour water contributed to epithelial lifting and necrosis in juvenile flounder.

*Chloride and mucous cell proliferation*

There was no significant increase in mucous cell numbers in juvenile flounder exposed to either ionic copper or Macquarie Harbour waters. This test endpoint was not sensitive enough to detect copper toxicity in these waters.

There was a significant increase in the number of chloride cells in the highest copper concentration tested (160 μg Cu/L) compared with controls at alpha = 0.05 by Dunnett's test. There was also a significant increase in chloride cells in both concentrations of station 9 water tested, with no effect in station 14 water. Again, dissolved copper concentrations in station 9 water were not high enough to explain the histopathological changes observed.

*Cloudy swelling in liver*

Large, swollen, faintly granular cells were present in livers of fish exposed to 50% and 100% Macquarie Harbour water from station 9 and 100% Macquarie Harbour water from station 14, as well as in the flounder exposed to 5, 20 and 40 μg Cu/L (fig 4.6). The prevalence of this change was significantly greater in the fish exposed to 100% Macquarie Harbour water (both station 9 and 14) compared with controls.

The content of these cells was PAS negative excluding the possibility of glycogen storage which can have similar appearance and would be normal. Because these swollen cells were not present in control fish and the material inside was not glycogen, they were presumed to be degenerated cells, possibly due to copper interference with normal cell functions. It is possible that it was due to failure of the sodium pump mechanism at the cell membrane, leading to accumulation of intracellular fluid. This type of cellular damage is called 'cloudy swelling'.

*Other effects*

The fish exposed to 80 and 160 μg Cu/L and some fish exposed to 100% Macquarie Harbour water from station 14 also showed necrotic changes in their liver and kidney (fig 4.7).

On the basis of the prevalence of histopathological changes in juvenile flounder, the fish exposed to 100% Macquarie Harbour water from both sites were affected as much as the fish exposed to 160 μg Cu/L. This suggests that copper is not solely responsible for the effects of Macquarie Harbour water on juvenile flounder.

Similar histopathological changes after copper exposure have been found in other studies with rainbow trout and winter flounder. Respiratory epithelium lifting has been reported in the gills of rainbow trout (*Oncorhynchus mykiss*) exposed to 64 μg Cu/L for 48 h (Bilinski & Jonas 1973). Increases in the number of chloride cells in gills as well as necrotic changes in liver, kidney and haematopoietic tissues were shown in winter flounder (*Pseudopleuronectes americanus*) exposed to 320–560 μgCu/L for 29 days (Baker 1969).

**Figure 4.6** Cloudy swelling in liver of an exposed fish

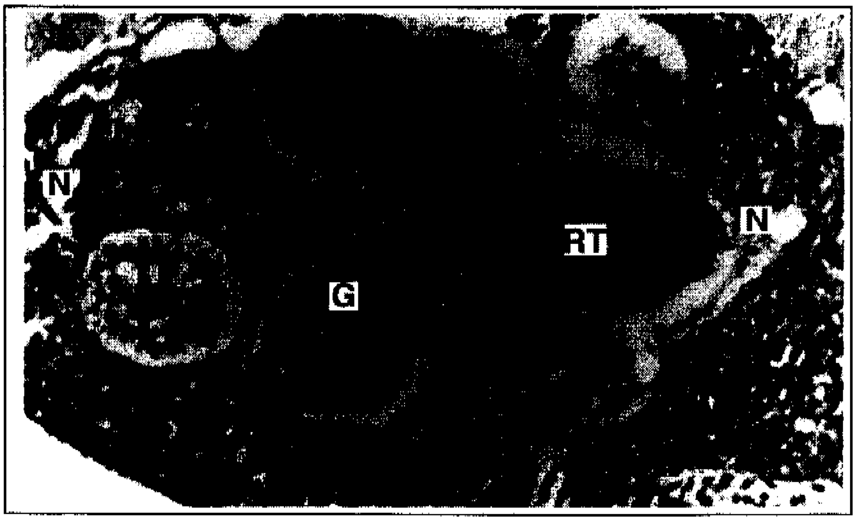



**Figure 4.7** Kidney of an exposed fish (N – necrosis, G – glomerulus, RT – renal tubule)

### 4.5.4 Freshwater challenge

There were no mortalities or any behavioural effects of freshwater challenge, indicating that 14-days exposure to Macquarie Harbour water from station 9 and 14 or to 5, 10, 20, 40 and 80 µg Cu/L does not affect osmoregulation in juvenile flounder.

### 4.5.5 Summary

Survival, osmoregulation and copper contents of juvenile flounder were not affected in 14-day exposures to Macquarie Harbour waters. Significant histopathological changes were observed, however, these were not attributable solely to copper. Other compounds or metals in the Harbour waters may be responsible for these effects.

Considering that increased salinity protects fish from copper poisoning, the greenback flounder seems to be sensitive to copper, relative to other species (Sorensen 1991). This may be due to the small size of the fish tested and their age or the species-specific differences in sensitivity to toxicants.

**Table 4.22** Prevalence of the most common histopathological changes in juvenile flounder after a 14-day exposure to ionic copper or unfiltered Macquarie Harbour water (stations 9 and 14)

| Treatment | Endpoint | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Epithelial lifting | | Epithelial necrosis | | Increase in chloride cells | | Increase in mucous cells | |
| | Prevalence (%) | CV (%) | Prevalence (%) | CV (%) | Prevalence (%) | CV (%) | Prevalence (%) | CV (%) |
| Control | 12.5 | 116 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 µg Cu/L | 38 | 39 | 31 | 77 | 6 | 13 | 0 | 0 |
| 10 µg Cu/L | 12.5 | 116 | 6 | 200 | 0 | 0 | 0 | 0 |
| 20 µg Cu/L | 38 | 39 | 13 | 116 | 0 | 0 | 6 | 13 |
| 40 µg Cu/L | 94[a] | 13 | 75[a] | 27 | 19 | 15 | 6 | 13 |
| 80 µg Cu/L | 81[a] | 30 | 69[a] | 35 | 14 | 20 | 6 | 13 |
| 160 µg Cu/L | 100[a] | 0 | 100[a] | 0 | 50[a] | 71 | 0 | 0 |
| Macquarie Harbour | | | | | | | | |
| Stn 9 (100%) | 81[a] | 30 | 88[a] | 116 | 38[a] | 52 | 6 | 13 |
| Stn 9 (50%) | 69[a] | 35 | 50[a] | 41 | 38[a] | 40 | 19 | 30 |
| Macquarie Harbour | | | | | | | | |
| Stn 14 (100%) | 94[a] | 13 | 75[a] | 82 | 19 | 30 | 12 | 17 |
| Stn 14 (50%) | 69[a] | 46 | 44[a] | 43 | 6 | 13 | 0 | 0 |

a The means for these groups were significantly greater than the control mean at $\alpha = 0.05$ by Dunnett's test

## 4.6 Risk assessment

Table 4.23 summarises the NOEC values for copper in Macquarie Harbour waters compared with clean seawater (20‰ salinity) for each of the bioassays used in the current study. Amphipod growth and algal enzyme inhibition were the most sensitive endpoints in Macquarie Harbour bioassays with NOEC values of <10 µg Cu/L. Algal growth was much less sensitive to copper in Macquarie Harbour waters than in clean seawater, due to adsorption of copper by organic and inorganic colloids in the harbour waters.

**Table 4.23** No effect values for copper in clean seawater (20‰ salinity) compared with Macquarie Harbour water

| Bioassay | NOEC (µg Cu/L) | |
|---|---|---|
| | Seawater | Macquarie Harbour water |
| Algal growth | 2.5 | >42 |
| Algal enzyme | <2.5 | <10 |
| Amphipod survival | >16 | >12 |
| Amphipod growth | 3 | 2, 6 |
| Flounder survival | 80 | >12 |
| Flounder histopathology | 20 | <10 |

A preliminary risk assessment of copper in Macquarie Harbour waters is given in Appendix A. This risk assessment compared recent monitoring data of ASV-labile, dissolved and total copper in mid-salinity waters of Macquarie Harbour with literature data on copper toxicity using lethal and sub-lethal end-points for a wide range of species in brackish, estuarine and marine waters. This assessment showed that all of the measured total copper concentrations (dissolved + filterable) were above the ANZECC marine water quality guideline value of 5 µg/L. There was a probability greater than 0.90 of the occurrence of ASV-labile copper at concentrations harmful to 5% of all species. For total dissolved copper the probability was higher than 0.98. It was concluded that dissolved copper in Macquarie Harbour would have to be reduced by a factor of 30 to provide sub-lethal protection for 95% of species for 90% of the time.

This preliminary risk assessment based on literature data for copper toxicity was necessarily conservative, largely because bioavailable copper was overestimated. Using the toxicity data from the algal and fish bioassays carried out in actual Macquarie Harbour waters, the probability of copper concentrations in the mid-salinity harbour waters exceeding no effect concentrations was reduced. However, it was not possible to estimate a single copper value which would protect against adverse effects in the Macquarie Harbour from the limited bioassay data in this study. It was possible to conclude that:

- Based on the algal growth bioassays in Macquarie Harbour waters, there was a probability of only 0.13 and 0.28 of the occurrence of ASV-labile and dissolved copper respectively occurring at concentrations above these values in Macquarie Harbour waters. The preliminary risk assessment showed that the copper concentration likely to be hazardous to 5% of algae was 15 µg/L, requiring a four-fold reduction in dissolved copper and a two- fold reduction in ASV-labile copper in Macquarie Harbour waters to protect 95% of the algal species 90% of the time. The algal growth bioassays however, showed no effect at copper concentrations as high as 42 µg Cu/L.

- Amphipod growth was more sensitive, with a probability of 1.0 that dissolved copper concentrations in Macquarie Harbour would exceed the NOEC of 3 µg/L. To protect this species of amphipod 90% of the time, dissolved copper concentrations would have to be reduced by a factor of 20. However, amphipod growth inhibition was only observed in one Macquarie Harbour sample tested (station 14) and poor recovery of controls was obtained in the copper calibration experiment. Further bioassays with this species would be necessary.
- Bioassays with flounder showed that there were no adverse effects on flounder survival, osmoregulation or copper accumulation at concentrations of copper found in Macquarie Harbour. Histopathological effects were observed in Macquarie Harbour bioassays but these effects were not solely due to copper.

Clearly, more toxicity data from a larger number of Macquarie Harbour samples are required to confidently assess the environmental impact of copper in these waters. Based on the risk assessment and data from the Macquarie Harbour toxicity tests, dissolved copper concentrations in mid-salinity Macquarie Harbour waters should be reduced at least four-fold. Dissolved copper concentrations of below 20 µg/L should have no adverse effect on algal growth, amphipod survival, or osmoregulation, survival and histopathology of flounder. Without the use of additional safety factors, we estimate that the maximum acceptable concentration for copper in Macquarie Harbour mid-depth waters lies between 10 to 20 µg/L. Effects on amphipod growth and algal enzyme levels were observed at lower copper concentrations, however the ecological significance of these endpoints is uncertain.

# 5 Conclusions

Chemical measurements of ASV-labile copper and copper complexing capacity of ten Macquarie Harbour mid-salinity waters (sampled in October and December 1995) have shown that copper concentrations in these waters exceed the complexing capacity of the dissolved organic matter in Macquarie Harbour. Similarly, a preliminary risk assessment comparing dissolved copper levels in Macquarie Harbour waters with concentrations of copper reported in the literature to have significant impacts on aquatic organisms, has shown that dissolved copper concentrations in Macquarie Harbour would need to be reduced. However, sensitive toxicity tests with algae, invertebrates and fish suggest that much of the dissolved copper in the Macquarie Harbour waters is not bioavailable.

Growth inhibition tests with the marine alga *Nitzschia closterium,* which can detect bioavailable copper at concentrations as low as 5 µg/L, showed that Macquarie Harbour waters containing up to 42 µg Cu/L were not toxic. Similarly, no significant increases in copper accumulation in juvenile flounder were observed after a 14-day exposure to Macquarie Harbour waters containing up to 12 µg Cu/L, even though significant increases in copper contents in fish exposed to ionic copper at similar concentrations were found.

No effect of Macquarie Harbour waters on amphipod mortality or juvenile flounder survival or osmoregulation were observed, although these bioassay endpoints were not sensitive enough to detect effects at copper levels environmentally relevant to Macquarie Harbour.

Small but significant effects of Macquarie Harbour waters on algal enzyme activity, amphipod growth (station 14 only) and juvenile flounder histopathology were found. However, histopathological changes in flounder could not be solely attributed to copper in the Harbour waters, because similar effects did not occur at equivalent low ionic copper concentrations.

Despite the limited sampling on two occasions only, it appears that much of the copper present in the Macquarie Harbour waters is not bioavailable. It is likely that colloidal metal oxyhydroxides in the waters reduce copper bioavailability, because electrochemical measurements showed that the waters (with up to 24 µg/L of copper ASV-labile) had no complexing capacity. Colloidal aluminium, manganese and iron oxides/hydroxides were present in sufficient concentrations to bind copper and reduce its uptake into organisms. Part of this fraction of copper may be detected as labile, but not able to dissociate at the cell membrane.

This work highlights the importance of using toxicity tests, not just chemical monitoring, to assess the impacts of pollutants in aquatic systems. Use of chemical analyses and literature data alone in this environmental risk assessment would have greatly overestimated the toxicity of copper in mid-salinity waters of Macquarie Harbour. Use of appropriate and sensitive toxicity tests has shown that only a small fraction of the measured copper is available to cause toxicity. A four-fold reduction in dissolved copper would be required to achieve acceptable copper concentrations of 10–20 µg/L in Macquarie Harbour mid-depth waters. More extensive sampling of Macquarie Harbour waters covering a broader range of salinities over different seasons would be required to ensure copper in these waters causes minimal impact on biota.

# 6 Recommendations for future work

Toxicity of only a limited number of water samples on two sampling occasions was determined in this study. Temporal and spatial variability in bioavailability and toxicity of copper in Macquarie Harbour waters is required with a more extensive sampling program.

This study focused only on the toxicity of mid-depth waters (20‰ salinity) to marine organisms. Further testing of surface, low-salinity waters which contain higher concentrations of copper should be carried out. To do this, suitable bioassays with estuarine species able to tolerate salinities as low as 5‰ should be developed.

The physico-chemical speciation of copper is critical to its toxicity in water bodies. More research is required to find the analytical speciation methods that correlate best with bioassays (eg algal assays) of copper-containing waters. At present, regulatory bodies base their criteria on *total* copper concentration, but many experts expect this to change in the future if reliable speciation techniques are developed and accepted. There is a similar need for correlation between copper speciation and toxicity in aquatic sediments.

There are very limited data on the toxicity of copper to marine fish. This is principally because marine fish are more difficult than freshwater fish to keep in culture. A survey of the chronic toxicity of copper to a range of early life stages and juvenile Australian marine fish would provide a data bank that would be useful to both industry and regulatory bodies in Australia.

Algal assays are particularly useful for assessing copper toxicity in waters, because they are relevant, sensitive and rapid. Unfortunately, much of the literature data on the toxicity of copper to various algal species are of limited value because the assays were carried out in culture media containing chemicals which complex copper and reduce its toxicity. More toxicity data need to be collected using natural waters as growth media. Also, it is evident that the susceptibility of a particular species of alga to copper can be quite different in an assemblage of algal species than if tested alone. More research should be carried out on the effects of copper on natural assemblages of algae. In addition, the toxicity of copper to algae is highly dependent on other metals present, and further work on the toxicity of metal mixtures to algae is required.

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## Appendix A

# Macquarie Harbour, Tasmania: Environmental risk assessment of toxic effect

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## 1 Introduction

As part of the larger program, this report compares the measured copper contamination of Macquarie Harbour with literature values of significant biological impact under marine or estuarine conditions. Due to constraints on available resources, this study should be regarded as preliminary in nature. Many of the data were not critically assessed according to quality criteria prior to acceptance for the risk assessment. The water quality data by their nature can only give 'snapshots' of copper concentrations at the moments of sampling. The biological assessment endpoints selected are also somewhat arbitrary, although they do encompass both lethal and sub-lethal parameters. However, this report will provide a good approximation of the degree of improvement in harbour water quality required to attain acceptable low levels of biological impact.

The aim of this risk assessment was to determine the degree of overlap between the distribution of measured concentrations of copper in water samples from Macquarie Harbour and the distribution of concentrations of copper reported in the literature to have significant effects on biota in marine or estuarine environments. By comparing these distributions, the probabilities of exceeding critical values of copper in the environment relevant to selected endpoints, such as proportional lethality to a prescribed range of species across trophic levels, can be determined within set confidence limits. We can then assess the generic risk that copper, in waters of specific harbour habitats, presents to biota likely to inhabit those regions.

The water quality distributions can also be used for comparisons with site specific ecotoxicological data determined for algae, crustaceans and fish, discussed elsewhere in this report (see section 4.6). As these values will be based on actual Macquarie Harbour waters, they will give a better indication of any synergistic or antagonistic influences on copper toxicity when compared with the predicted effects from the literature.

## 2 Experimental

Data used in the analyses are available from the authors.

### 2.1 Macquarie Harbour water monitoring data

Monitoring data for various stations within Macquarie Harbour were provided by Dr Lois Koehnken (DELM). The data comprised a comprehensive but incomplete (for a variety of reasons) set over the period from May 1993–August 1995 at approximately 3 month intervals. The incompleteness is mainly due to poor weather or low water levels at the time of sampling. Quality assurance checks on the electronic transfer of the information indicated that the data arrived safely.

Missing data were ignored. Stations 35 and above were sampled on only one or a few occasions, so these stations were excluded for general consistency between dates.

The data were arranged by analysis type, ie anodic stripping voltammetry-labile copper (ASV), total dissolved copper (hereafter referred to as dissolved) (µg/L) and particulate copper (mg/L or ppm). The ASV labile and dissolved copper values were determined after filtration (0.45 µm), whilst the particulate copper was determined from that retained on the filter. The mid-water samples were taken at the point at which 20‰ salinity was measured in the profile. This represented the middle of the salt wedge boundary between the deeper, more dense, seawater and the shallower, less dense, river water.

Mid-water data were selected for modelling. This selection coincided with the choice of this salinity for the ecotoxicological studies carried out within the project. The use of this water quality was based on the assumption that the marine species to be tested could tolerate these salinity conditions and also that copper input from the fresh waters would be both more concentrated and more soluble, and hence bioavailable, under these conditions than in the deeper, saltier waters. It was thus inferred that these conditions would give the most conservative assessment of copper toxicity in the Harbour.

Within this category, ASV-labile and dissolved copper were selected for distribution analyses. Dissolved copper represents the upper extreme of measurable copper likely to be toxic. On the other hand, ASV-labile would more closely represent the bioavailable fraction, but by its nature this measure will still tend to overestimate toxicity (see following discussion) and as such is still an ecologically conservative estimate.

Total copper, the sum of dissolved and particulate copper, was also derived to compare with the ANZECC (1992) guideline values.

### 2.2 Biological data

Literature values were taken from this report and the pre-1980 review by Hodson et al (1979). Only criteria specific data were selected, that is, marine or estuarine, LC values for lethal endpoints and lowest or no observable effect concentrations (LOEC, NOEC) or EC values for sub-lethal effects. Algal toxicity data from experimental studies carried out in full nutrient media, containing compounds that absorb or complex copper, reduce copper toxicity and thus underestimate its effect (Stauber pers comm). These data were therefore excluded. Given the resource constraints on the study, no other quality criteria, such as listed in Emans et al (1993), were applied to reject literature data. Identical data from both review sources were included once only.

The data were arranged by broad taxonomic group, ie algae, crustaceans, molluscs, and a few other smaller invertebrate groups, fish, and combinations of marine invertebrates and all marine taxa. The data within each category were separated into lethal (LD50 and LC50) and sub-lethal (EC50, LOEC and NOEC) criteria. In some categories not every criterion was available.

### 2.3 Statistical analysis

Preliminary observation showed that the water concentrations and subsets of the biological effect data were biased towards higher copper concentrations. In some cases this was extreme. Because of this, the data were assumed to be log-normally distributed and geometric means and standard deviations were derived. This form of distribution is typical for data of this nature (eg Kooijman 1987). Probability distribution functions were generated using these statistics within the STATISTICA software package (Statsoft Inc 1994). The goodness of fit of each derived log-normal distribution was determined using the Kolmogorov-Smirnov one sample test or the Chi-Squared test (Steel & Torrie 1981) at a significance level of 5%. The extreme high values mentioned earlier did not allow an adequate fit to the log-normal model. Thus, these values, which can be considered as outliers, were excluded in order to achieve statistically significant goodness of fit for the biota distributions. This action will make the assessed risk more conservative as the species most tolerant of copper pollution have been excluded in favour of more sensitive taxa.

Assuming that 5% of the representative population could be affected (ie a protection level of 95%), the critical hazardous copper concentrations (HC5%) for each of the subsets of biota distributions were derived (Wagner & Lokke 1991). The 95% and 50% confidence intervals around these estimates were also determined as per Aldenberg & Slob (1993). These values were then imposed on the distributions generated for the water sample copper concentrations (ASV and dissolved) to determine the prevailing probability of exceeding the critical water concentrations and also the degree by which water concentrations would need to be reduced in order to achieve the nominated degree of protection.

## 3 Results and discussion

### 3.1 Copper water concentrations

The selected water concentrations were observed for any seasonal and other temporal trends in their maximum, minimum and average values at stations for each sampling period (figs A3.1 a, b and c). Despite the occurrence of occasional high values, that may reflect sediment disturbance or increased pollutant inflow from the King River due to storm activity, there were no persistent patterns over the period of monitoring. These observations imply that copper concentrations, in this specific compartment of the areas affected by pollution from Mt Lyell, are currently relatively constant. On this basis, all further comparisons in this report used data combined from all sampling times.

### 3.2 Comparison with water quality guidelines

Figure A3.2 shows the degree of overlap between measured Macquarie Harbour copper concentrations and the ANZECC (1992) guideline values of total copper (dissolved plus particulate) for the protection of marine ecosystem health. None of the measured total copper concentrations were less than the guideline value of 5 ppb (0.7 on the log10 scale) which is commonly taken as the default regulatory limit. Even dissolved copper (the typical measure of environmental copper concentrations in water) and ASV-labile copper (a value more closely approximating bioavailable concentrations) were in excess of the guideline value most of the time. More than 95% of the measured dissolved copper values and 75% of the ASV-labile values were greater than the guideline at all times.

Total copper is not a good measure of environmental hazard as most of the measured metal is not biologically available and as such will not directly contribute to toxic effect. Indirect contribution is possible depending upon the degree to which the particle associated copper can be mobilised into a bioavailable form. Exceptions to this generalisation include toxicity from particulate copper to members of the ecological community that are filter feeders, detritivors that ingest particles containing copper, and plants that use the copper bearing particulates as a nutrient substrate. These exposure pathways can be especially significant if the affected taxa include keystone species.

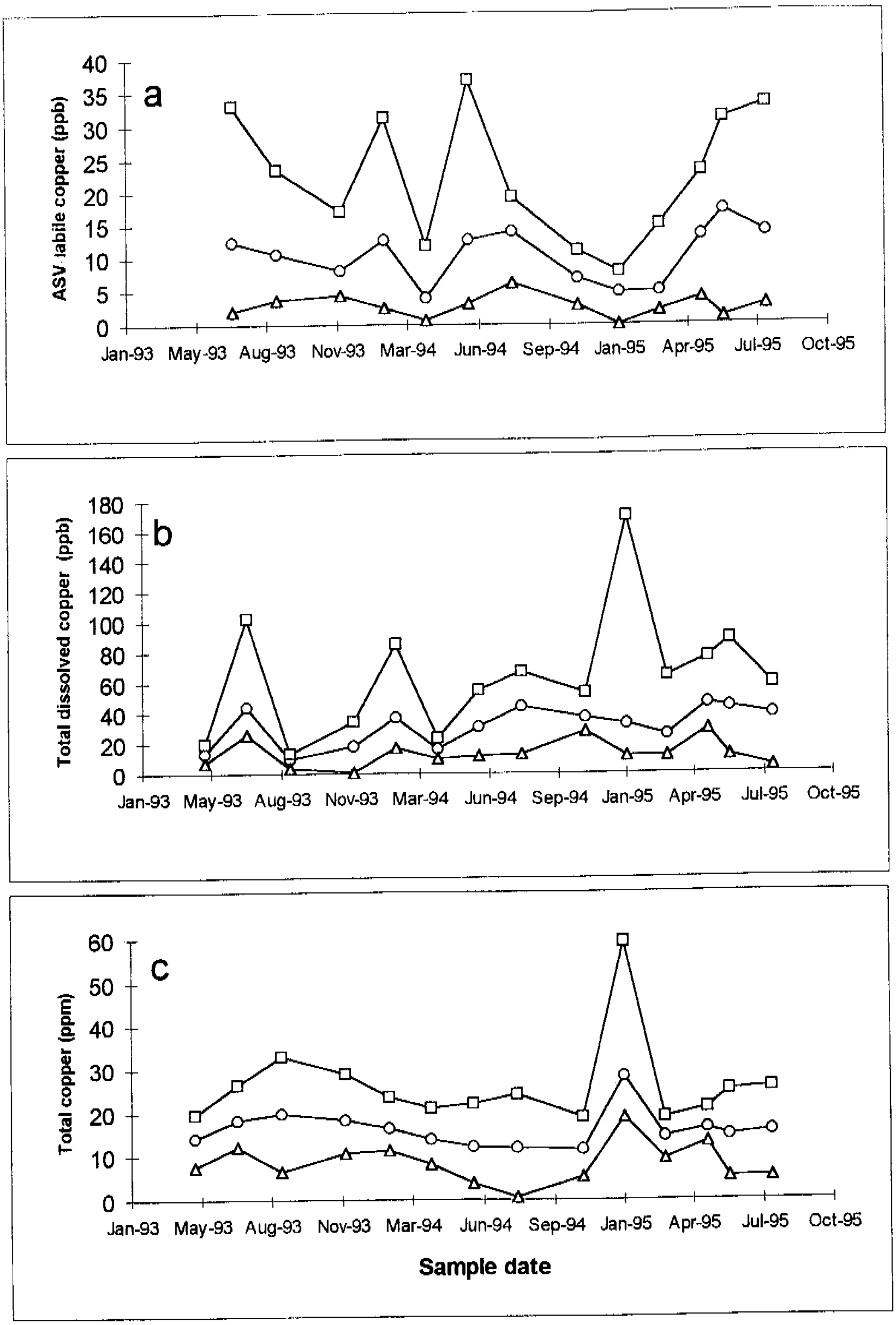


**Figure A3.1** Maximum (□), average (○) and minimum (Δ) copper concentrations in mid-salinity Macquarie Harbour waters at each sampling period as measured by: a) anodic stripping voltammetry-labile copper; b) total dissolved copper (0.45 μm) (both in μg/L); and c) total copper (dissolved plus filterable) (mg/L)

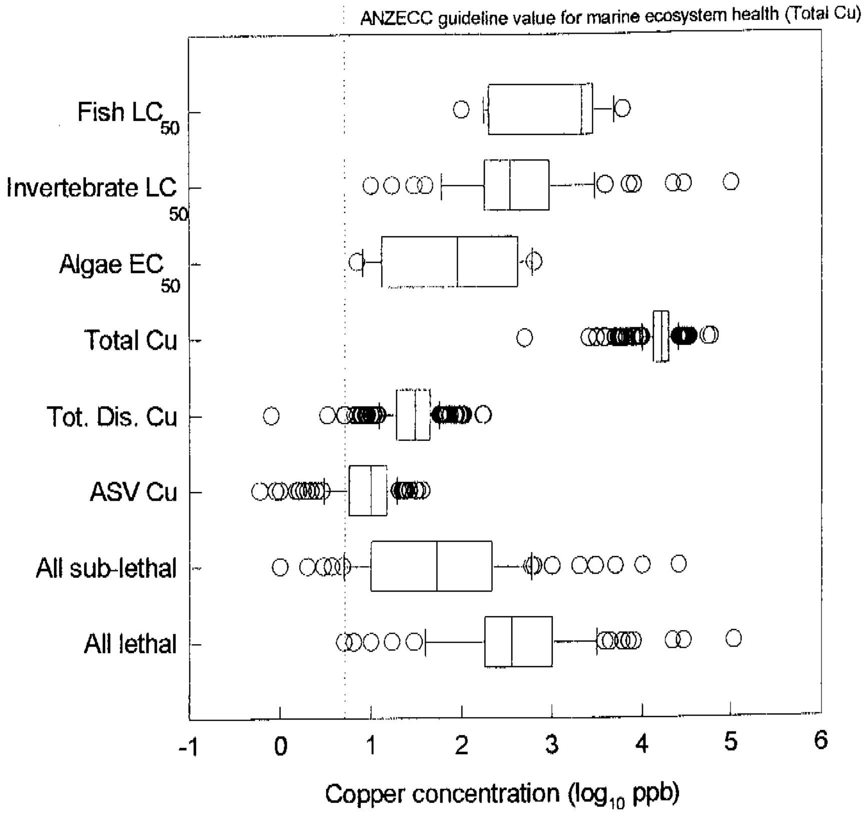


**Figure A3.2** Box plots of measured copper concentrations at mid-salinity depths of selected Macquarie Harbour sampling stations (see text) and sub-sets of raw ecotoxicity data (lethal and sub-lethal) from the literature in relation to the ANZECC guideline copper concentration for marine ecosystem health (dotted line). The boxes extend from the 25th–75th percentiles with the median as a mid-line. The capped bars indicate the 10th and 90th percentiles and symbols indicate data outside these values.

### 3.3 Biological data

It is generically assumed that the concentrations for each of the biological endpoints used in the data sorting will decrease in the order LC50>LD50>EC50≥LOEC>NOEC. Chronic NOECs were found to be 10–30 times lower than acute median lethal values on average by Hendricks (1995) when studying organic toxicants. This general pattern could be observed in the raw data of our current study, particularly where results for a single species or within closely related taxa were examined. However, this was not always found to be the case as some of the observed sub-lethal criteria were less sensitive than others and there were wide ranging degrees of tolerance between species. That is, some very tolerant organisms showed no or low observed response to very high concentrations of copper (high NOEC/LOEC) whilst some extremely sensitive organisms died at low concentrations or exposures (low LC50, LD50).

In fig A3.2 the measured copper concentrations in Macquarie Harbour waters are compared with sub-sets from the literature data indicating the biological effect of copper. Both lethal and sub-lethal parameters are represented. The plots of All lethal and All sub-lethal data include information in addition to that given for the sub-sets at the top of the page.

The available literature data cover several trophic levels. The information density varies between these levels but the discrepancies are minor. Also, the biological effects within any category occur over orders of magnitude differences in copper concentrations. From these observations it is apparent that the data have provided a representative spread of effect levels for both sensitive and insensitive species across most trophic levels and, as such, they provide a reasonable basis for ecosystem scale assessment.

The boxplots representing all lethal and sub-lethal data show a substantial overlap (fig A3.2). However, sub-lethal effects may be seen to generically occur at copper concentrations an order of magnitude lower than those observed for lethal effects.

The probability distribution functions of combined taxa lethality data (LC50 and LD50 values) and sub-lethality data (EC50, LOEC and NOEC values) are shown in figs A3.3a and b respectively. The most sensitive comprehensive subset, algal EC50 data, could not be adequately fitted by a log-normal model.

From the raw data, the copper concentrations likely to be hazardous to 5 and 10% of the biota at the given endpoint are given in table A3.1. Also given are the parameters derived from the fitted distributions. These are the HC5% and the lower limits of the 50% and 95% uncertainty, or confidence, ranges about the critical value.

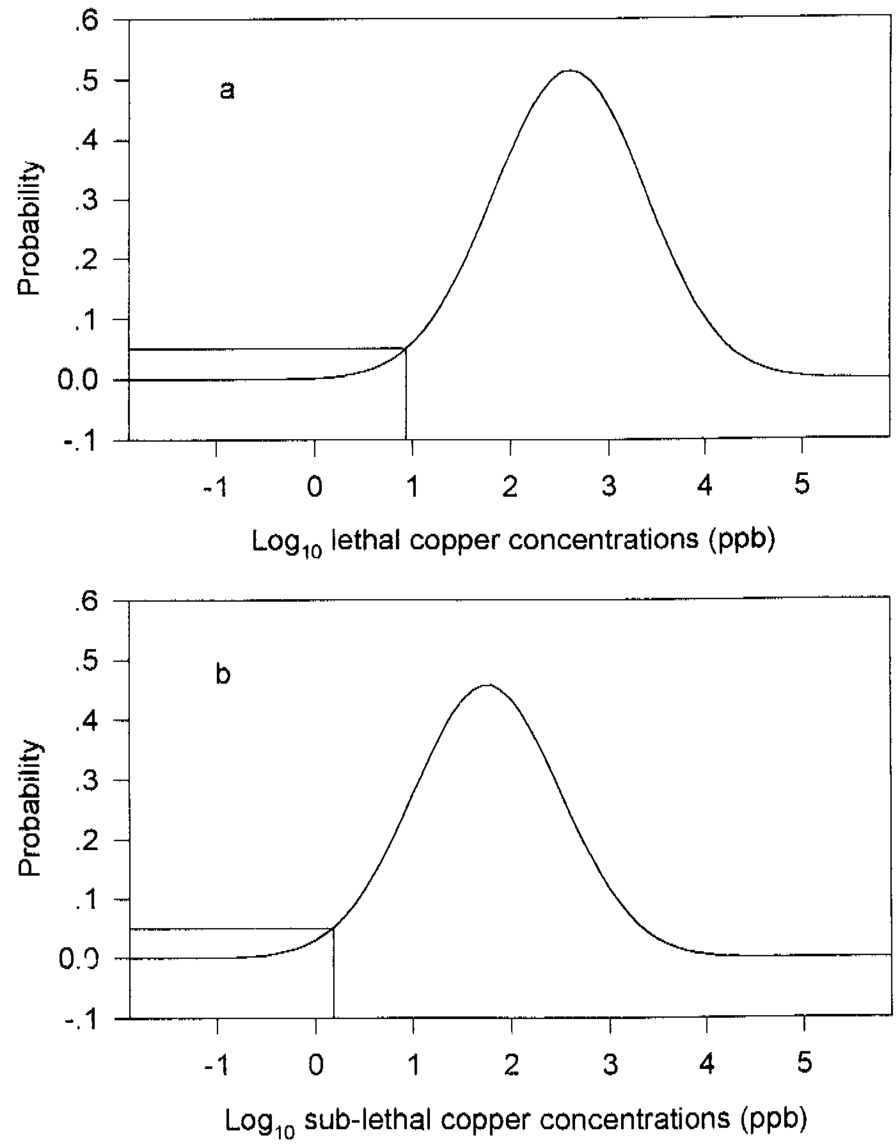


**Figure A3.3** Probability distribution functions of copper toxicity data for a) lethal and b) sub-lethal end-points taken from the literature. Extreme (high) values were excluded to allow statistically adequate fit of the distributions. The intercepts indicate the copper concentrations below which only 5% of species are predicted to show a response.

**Table A3.1** Critical values of copper concentrations (ppb) that have lethal and sub-lethal effects on biota. Outliers were high values which were removed from the data sets to allow for statistically adequate model fitting.

| | | Raw data | | | Fitted distribution | |
|---|---|---|---|---|---|---|
| Taxonomic group | Criteria | 5% | 10% | $HC_{5\%}$ | 50% conf. value | 95% conf. value |
| All | lethal | 30 | 60 | | | |
| All (- outliers) | lethal | 17 | 40 | 21.1 | 20.8 | 9.4 |
| All | sub-lethal | 4.9 | 5 | | | |
| All (- outliers) | sub-lethal | 3.7 | 5 | 2.1 | 2.1 | 0.9 |
| algae | sub-lethal | 15 | 15 | | | |
| invertebrates | lethal | 17 | 40 | 23 | 21 | 5.1 |
| fish | lethal | 100 | 200 | 93.8 | 84.9 | 18.1 |

### 3.4 Comparison of the water and biota distributions

Predominantly the literature data refer to soluble or dissolved copper concentrations, particularly when dealing with determination of lethal endpoints. Hence total copper concentrations (dissolved + particulate) provide a poor basis for comparison. Field data in particular refer mainly to dissolved copper concentrations. Experimental data are generally concerned with ionic copper species and therefore more closely correspond to the ASV-labile values.

Thus, for comparison of likely toxic effect, dissolved copper will give the upper limit to possibly toxic copper concentrations in Macquarie Harbour. However, Macquarie Harbour waters are known to have a very high complexation capacity, predominantly from the levels of organics input from the surrounding freshwater catchments. From this, it is reasonable to refer to the ASV-labile copper distribution for a more realistic appraisal of ecological risk. This assessment will still be conservative as the copper measured as ASV-labile will include species such as carbonates that are non-toxic (Hunt 1987) and copper that is moderately bound to some organic ligands within the water column (Batley pers comm). These components of the measured copper are not considered to be biologically available. The cumulative probability distributions of ASV-labile and dissolved copper in Macquarie Harbour mid-salinity water samples are shown in figs 3.4a and b respectively.

Using the 5% value in the raw data across all species (less outliers) for comparison with the distributions derived from the monitored copper concentrations in Macquarie Harbour, it can be seen that the current probability of exceeding the lethal critical concentration is 0.76 based on the dissolved copper and 0.19 based on the ASV-labile copper. The lower 50% confidence interval of the HC5%, based on the distribution with outliers removed, is less restrictive. The likelihood of exceedence in these cases reduces to 0.66 and 0.12 for the two measures of copper concentration respectively.

The sub-lethal HC5% lower 50% confidence limit, derived excluding outliers, is exceeded with a probability of 0.98 (almost all the time) based on the ASV-labile distribution and of approximately 1 (ie at all times) using the dissolved copper. The 5% point in the raw data is exceeded with a probability of 0.90 and 1.00 by the two measured copper distributions respectively. If Macquarie Harbour is assumed to have similar proportions of copper in biologically available forms to waters tested in the literature then the 0.90 probability concentration of total dissolved copper in Macquarie Harbour would need to be reduced by a factor of approximately 30 to achieve adequate protection based solely on this criteria without application factors.

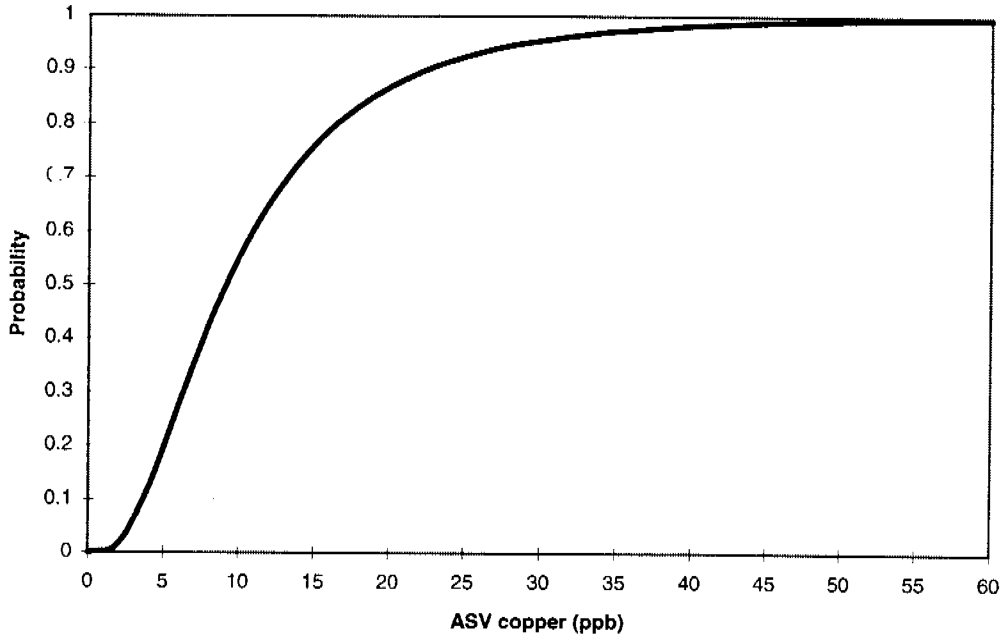


**Figure A3.4a** Cumulative probability distribution of ASV-labile copper in mid-salinity waters of Macquarie Harbour. The curve indicates the probability of measuring a concentration less than any specified value, based on monitoring data.

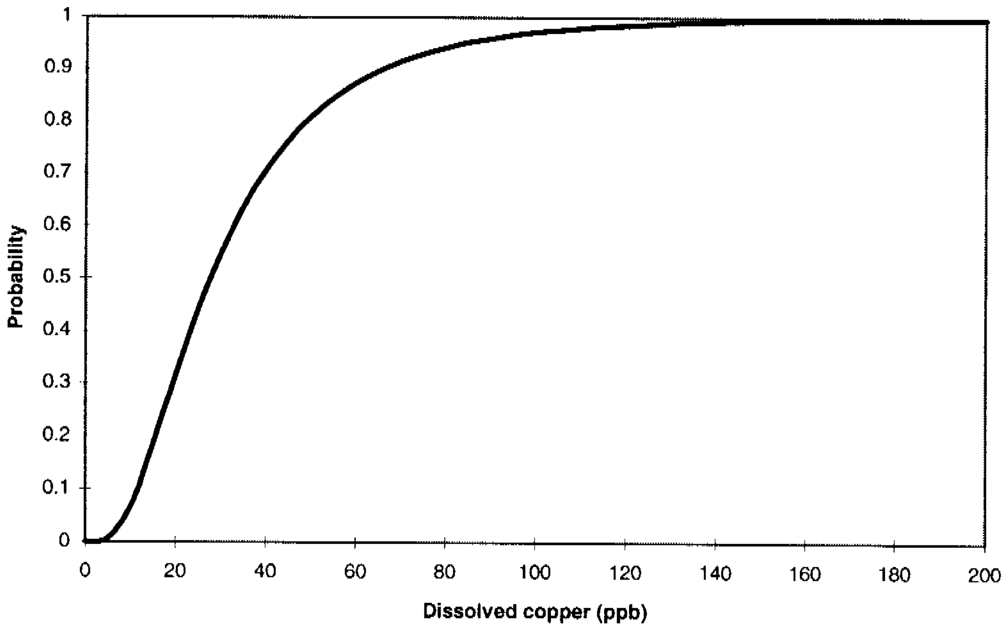


**Figure A3.4b** Cumulative probability distribution of total dissolved copper in mid-salinity waters of Macquarie Harbour. The curve indicates the probability of measuring a concentration less than any specified value, based on monitoring data.

The most sensitive 5% of algae are currently exposed to sub-lethal concentrations with a probability of 0.24 based on the raw data and ASV-labile water concentrations. To protect 95% of the algal species 90% of the time a reduction in ASV-labile copper concentrations by a factor of approximately 2 is required.

When making assessments of the degree of reduction in copper required to achieve this type of end-point, the degree of dissolution from sediment must also be taken into account in addition to reductions in input via the King River.

The lethal parameters for invertebrates and fish are included in table A3.1. The raw data are also shown in fig 3.2. It can be seen that invertebrates are relatively comparable to the generic lethal data and, as such, sub-sets of crustacean data may be a reasonable surrogate for more extensive biological data in other comparisons. The fish data are relatively insensitive when compared with the endpoints derived for combined taxa lethal data or any sub-lethal parameters.

### 3.5 Factors that affect the relative degree of safety implicit in the risk analysis

The following factors provide inherent conservatism (safety) to the risk assessment:

*Use of measured values that overestimate the biologically available copper water concentrations.*

The labile copper measured by ASV as well as the dissolved concentrations include some chemical species that are less, or not, bioavailable. These chemical species are less toxic than the free ionic form of copper.

*Use of mid-salinity water quality data maximised the measured copper concentration in the marine waters of Macquarie Harbour and, hence, the perceived risk to marine organisms.*

*Use of all water measurements rather than averages for any period.*

There is little likelihood of all sites being simultaneously contaminated to high levels. The average values at any particular sampling period (fig A3.1) indicate a distribution about a factor of two lower than the maximum values measured at the same time. This provides an additional safety factor in the assessment given that motile species will be at an advantage in that they may avoid or move out of highly contaminated zones to other depths or locations, and widely distributed species will be able to recolonise affected areas.

*The data from the literature studies represent effects to proportions of individuals within populations rather than to populations as a whole.*

Higher concentrations would be required to affect all individuals within the tested populations.

*Probable bias in the literature data towards sensitive species.*

Research workers will tend to select species that are most likely to show a significant response to any test. Sensitive species are also likely to have been chosen for testing or monitoring on the basis of their relative response in field surveys. In addition, the exclusion of extremely tolerant species from the biota data to achieve normal distributions has biased the data towards more sensitive taxa.

*The use of laboratory studies to estimate environmental risk.*

Most controlled laboratory studies constrain the experimental parameters to minimise variability. Many natural water quality parameters that reduce toxicity (eg complexation capacity) are thereby excluded from these studies. Hence, this may lead to an overestimate of toxic effect when the results of laboratory studies are applied to natural systems.

*The use of the lower limit of the uncertainty estimate of the critical hazardous concentration is inherently conservative.*

*The likelihood of the occurrence of tolerant populations of species within the Harbour brought about by over a century of natural selection pressure.*

**The following factors are of unknown significance or could contribute to an estimate of greater risk from copper to biota in Macquarie Harbour:**

*The magnitude of significant water quality parameters*

This study has looked solely at copper concentrations in the mid-salinity habitat of Macquarie Harbour. There has been no specific attempt in this study to address the other habitat parameters that can influence copper toxicity. These include possible synergistic effects from other toxic materials (eg zinc) and antagonistic effects such as complexation by organic ligands or the formation of non-toxic metal species.

*The keystone species for ecological sustainability have not been identified.*

At present too little is known of the local biological communities, either within Macquarie Harbour or in similar habitats unaffected by the pollution from Mount Lyell. As such the keystone or indicator species have yet to be adequately identified for the overall study. The successful identification and re-occurrence of these species within Macquarie Harbour is certain to be one of the criteria for success of the overall remediation process.

*The impact of copper concentrations in the upper water layer habitat of Macquarie Harbour has not been addressed.*

Very high levels of copper are present in the less dense river water suspended above the saline wedge within the Harbour. The copper levels are well in excess of the ANZECC guidelines for freshwaters. The impact on euryhaline, migratory or freshwater species could be significant at the measured concentrations. Any assessment of this habitat should include toxicity to water fowl including bioaccumulation pathways.

*Bioaccumulation pathways and their associated risk, to other biota or humans, have not been addressed.*

*Quality criteria have not been applied to the selection of literature data used for the models.* Exclusion of data will lead to changes in the probability distributions but the significance of these changes cannot be assessed at present.

*Sediment effects*

Estimations on the degree of copper concentration reductions required will need to include an assessment of the likely remobilisation of sediment bound copper as well as reductions in riverine input. It must also be recognised that some keystone organisms, environmentally critical to the remediation, may occupy a benthic habitat. As such, these species will be at risk from current and future sedimentary copper.

## 4 Conclusions

When compared with the ANZECC (1992) water quality guidelines, copper concentrations in Macquarie Harbour waters are too high. This preliminary comparison justifies the need for a more comprehensive risk assessment.

When alternate, less restrictive, criteria are used to compare concentrations of copper in Macquarie Harbour water with literature data on the biological effects of copper in marine systems, the monitored water concentrations still exceed the critical hazard levels using both lethal and sub-lethal endpoints. Based on these more realistic evaluations the prevailing total dissolved copper water concentrations have a probability of 100% of exceeding the sub-lethal critical limit and of 66% of exceeding the critical limit for lethality.

If Macquarie Harbour is assumed to have similar proportions of copper in biologically available forms to waters tested in the literature, then to achieve water concentrations that have an adequately low probability (eg 10%) of exceeding the critical sub-lethal limit across all taxa, a reduction in total dissolved copper water concentrations by a factor of at least 30 would be required. However, the results obtained for Macquarie Harbour waters indicate that the actual toxicity is considerably lower than would be expected from the copper concentrations measured. For algae, important as the autochthonous primary producers of the ecosystem and the most sensitive taxonomic group, the reduction of the ASV-labile copper concentration in water that is required to protect 95% of species is an approximate factor of only 2 based on the available literature.

It must be stressed that the risk assessment is reasonably conservative for a variety of reasons (see 3.5). Predominant amongst these are that a relatively low risk of hazard (5%) was chosen as the critical assessment level; that sub-lethal endpoints were considered; and that bioavailable copper was over estimated. Use of toxicity data from bioassays carried out in Macquarie Harbour waters should enable better prediction of the copper concentrations able to be tolerated by aquatic organisms in this harbour.

Factors that may contribute to risk but which have not been addressed in this study include the possible presence of metals such as zinc that act synergistically with copper. It is also imperative that a biological survey be undertaken to identify, if possible, potential keystone species in equivalent environments with particular note of any filter feeders or benthic species that may be affected by the high concentration of copper in the Harbour sediment.

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