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 ($CuCO_3^o$, 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] 0.05[d]	-[c] 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 °/oo	Stauber & Florence (1989)	10
	f	Stauber & Florence (1989)	200
Phaeodactylum tricornutum	sw, 35 °/oo	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 °/oo	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 Chlorococcocales 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(‰)	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)	
(‰)	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 control 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 cucullata* 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 ± 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 3×10^{-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.

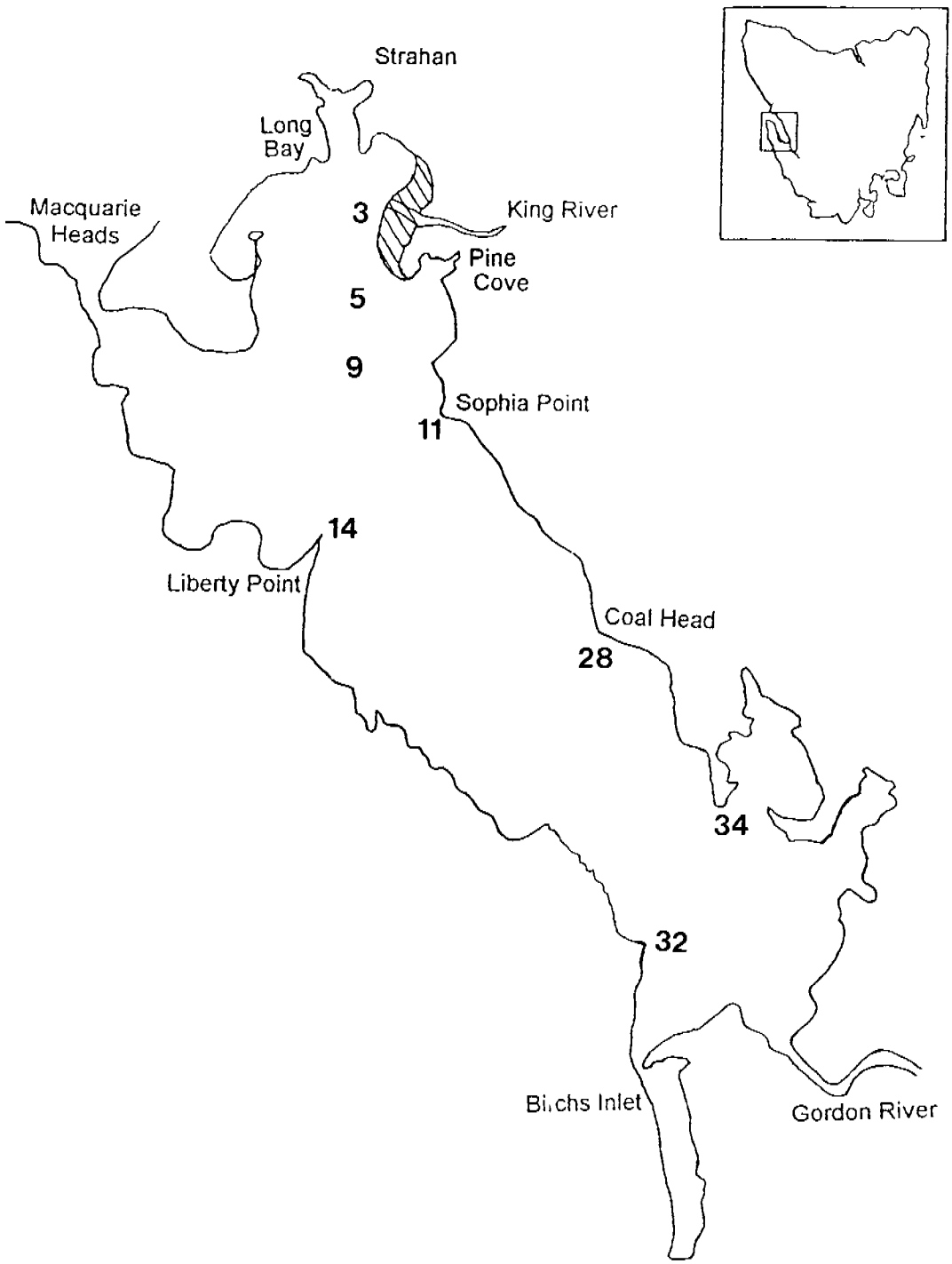

Figure 3.1 Field collection sites in Macquarie Harbour

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 (°/oo)	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%).