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Copper speciation and toxicity in a contaminated estuary

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Executive summary

This report describes a brief study of the relationship between chemical and biological estimates of toxicity in a contaminated estuarine waterbody. A suite of tests was conducted using organisms chosen to represent several trophic levels and taxonomic groups. Four species were screened for suitability including the marine alga *Nitzschia closterium,* the sea urchin *Heliocidaris erythrogramma,* the rotifer *Brachionus plicatilus*, and the brine shrimp *Artemia salina*. The marine alga was found to be the most suitable species for testing, due to the ease of culture, sensitivity to copper, and reliability and repeatability of the tests.

Copper speciation was investigated using Anodic Stripping Voltammetry (ASV-labile copper), free copper by Ion Selective Electrode and total copper to determine if copper toxicity of estuarine samples could be predicted from chemical parameters. Algal tests were used to investigate copper speciation and toxicity in samples collected fom Macquarie Harbour, a contaminated estuary in Tasmania. *Nitzschia closterium* showed a strong correlation between toxicity and free copper in laboratory studies using clean seawater. No toxicity was observed in the field samples, despite significant levels of 'bioavailable copper' measured by ASV. Potentiometric measurements of copper showed there was insufficient free copper present in the samples to cause toxicity. Free copper measurements, in conjunction with other speciation techniques and bioassay data, can contribute to a better understanding of bioavailability and toxicity to aquatic organisms.

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Copper speciation and toxicity in a contaminated estuary

RS Eriksen, B Nowak & RA van Dam

1 Introduction

1.1 Copper speciation and toxicity

Copper is an essential nutrient for aquatic organisms, but can also be toxic at elevated concentrations (Sunda & Hanson 1987). Speciation plays a critical role in determining if copper is biologically available, toxic, or unavailable. In natural waters, copper and other trace metals will be complexed to both organic and inorganic ligands, so that the concentration of the free metal ion may be many orders of magnitude lower than the total metal concentration. The free metal ion is more chemically reactive than complexed forms of the metal, and hence is believed to be the most biologically available form (Sunda & Hanson 1987).

Estuaries are dynamic environments with respect to metal speciation, as many of the physico-chemical parameters that affect metal equilibria are subject to change as fresh and marine waters mix. pH, hardness, dissolved organic matter concentration, and ionic strength will all significantly affect the partitioning of copper within and between solid and solution phases (Depledge et al 1994). This in turn can strongly influence the uptake of the metals by aquatic organisms.

Open oceans have very low concentrations of copper, typically 0.12–0.38 μg/L (Mackey 1983). The concentration of copper in estuaries is usually higher than open ocean, due to the contribution of terrestrial sources (eg runoff), inputs from rivers, and domestic effluents and urban stormwater (Depledge et al 1994). Macquarie Harbour, on Tasmania's west coast, has numerous inputs from such sources, but by far the greatest contributor of heavy metal contamination is the Mount Lyell copper mine at Queenstown. For the past century, mining operations have resulted in the dumping of tailings, smelter slag and topsoil into the Queen and King Rivers, which eventually flow into the harbour (Koehnken 1996). Copper concentrations up to 1000 μg/L have been reported in plumes of contaminated water from the King River. The harbour presents an interesting scenario because previous studies have shown that chemical tests of 'bioavailable' metal predict significant toxicity (Stauber et al 1996). In stark contrast, biological assays using a variety of organisms showed little or no toxicity (Stauber et al 1996). Macquarie Harbour is an estuary where complex chemical and biological relationships exist, and the mechanism of amelioration of toxicity has only recently been investigated (Stauber et al 2000).

The aim of this research was to investigate the relationship between toxicity and copper speciation in a range of estuarine organisms. Four species were chosen for initial screening: the estuarine diatom *Nitzschia closterium*, a local sea urchin *Heliocidaris erythrogramma*, the rotifer *Brachionus plicatilus*, and the brine shrimp *Artemia salina*. These organisms vary in their sensitivity to copper, thus potentially offering a suite of practical tests, which ideally could be selected depending on the degree of copper contamination encountered. Different groups of invertebrates are represented, and different life stages and endpoints were used for

each test. While it makes the comparison between copper sensitivity of different species very difficult, the choice of life stages and endpoints reflected the ones most commonly used in toxicity testing.

1.2 Analytical techniques

Three chemical techniques were used to measure copper speciation in laboratory studies: an electrochemical measurement of labile copper using anodic stripping voltammetry (ASV), a potentiometric measurement of free copper by ion-selective electrode (ISE) and a spectrophotometric measurement of total copper using graphite furnace atomic absorption spectrometry (GFAAS). Traditionally, total metal has been measured to monitor copper levels in environmental samples. However, it has been suggested that ASV copper measurements better reflect the levels of bioavailable copper. ASV measures both the free metal ion in solution and any labile copper complexes or colloidal particles that dissociate or are directly reduced at the electrode surface (Sunda & Hanson 1987, Florence 1989). Free copper measurements by ISE at the concentrations found in brackish and marine waters have been developed and validated only recently (De Marco 1994, Mackey & De Marco 1997, Zirino et al 1998, Eriksen et al 1999).

1.3 Invertebrate bioassays

Marine and estuarine species commonly used for testing the toxicity of chemicals and for assessing environmental quality include rotifers, crustaceans, brine shrimps, mysids and bivalves (Widdows 1993). In general, the selected species are available at low cost, easy to maintain in the laboratory, have been used by other laboratories, and (ideally) have commercial or ecological importance (Widdows 1993).

1.3.1 Sea urchin fertilisation test

Early life stages of marine invertebrates have frequently been used for determining the effects of environmental contamination. The sea urchin bioassay used in this study was based on the protocols developed for the Australian urchin *Heliocidaris tuberculata* (Simon & Laginestra 1997). The bioassay tests for reduced reproductive viability by exposing sperm to the test contaminant, and then determining fertilisation success of eggs mixed with the exposed sperm. The test was developed for assessing sub-lethal toxicity of pulp mill effluents for the National Pulp Mills Research Program, and abnormal development may also be used as a test endpoint. Sea urchins have been widely used for toxicity testing of marine effluents, providing short-term, sensitive and relatively inexpensive methods for assessing aquatic toxicity (Zuniga et al 1995). Fertilisation success can be determined microscopically within minutes of mixing eggs and sperm suspensions, making the assay rapid. *Heliocidaris tuberculata* is not found in Tasmanian waters, so an alternative species of urchin, *Heliocidaris erythrogramma*, was investigated. *H. erythrogramma* has been used previously in Australia for toxicity tests on heavy metals (Kobayashi 1980), although tests have not been conducted using individuals collected from Tasmanian waters. The major disadvantage of this bioassay is that each sample must be adjusted to salinity 35 with hypersaline brine. Therefore the more brackish samples will be diluted significantly, and it will not be possible to test a sample at 100% strength. Ideally, urchins in spawning condition should be available all year round. In Tasmania, this is only possible with *H. erythrogramma* if the urchins are maintained in a culture facility.

1.3.2 Brine shrimp test

Artemia salina forms dormant cysts that can be easily hatched and used for toxicity tests (Widdows 1993). The advantages of this test include wide salinity tolerance of this species (5–150 ppt), short exposure time, availability of the cysts, low cost and simplicity of the test. Unfortunately, the endpoint is difficult to define and the brine shrimp is generally insensitive to toxicants (Widdows 1993). Snell and Persoone (1989) reported a 24 hr LC_{50} of 4.9 mg/L for copper in acute lethal toxicity tests using brine shrimp. The commercial and ecological significance of the species is considered to be low (Widdows 1993).

1.3.3 Rotifer test

Brachionus plicatilus is used widely as a food source for juvenile fish in aquaculture establishments (Nichols et al 1996). Advantages of the test are wide salinity tolerance, easy to determine biological endpoint, short exposure times, low set up costs, and the simplicity of the tests. The major disadvantage is the poor sensitivity of the test, with effects being detected at 'environmentally unrealistic' concentrations. The commercial and ecological significance of the species is considered low (Widdows 1993). *Brachionus* are more sensitive than brine shrimp, with a 24 hr LC_{50} for copper of 0.13 mg/L, but this is still not sufficiently sensitive for widespread applications (Widdows 1993). Cultures were obtained from the Tasmanian Aquaculture and Fisheries Institute (TAFI) at Taroona, Tasmania, as it was not possible to obtain cysts. Rotifers are bred in a continuous culture system, with 0, 1, 2 and 3 day old organisms present in the one culture (P Hart, pers comm). Cultures producing organisms of the same age were not available at the time of the study.

1.4 Algal bioassays

Algal bioassays have been used successfully for the assessment of metal toxicity in marine and estuarine waters (Walsh 1993). They have a number of distinct advantages over other test organisms. Microalgae are primary producers and therefore have a fundamental role in the health of aquatic environments. The impact of metal toxicity on algae may therefore have a significant effect on higher trophic levels. Standardised algal bioassays are rapid, with exposure times between 24 and 72 hours, and growth inhibition of algae is a sensitive indicator of toxicity. Additionally, microalgae can be used to determine sub-lethal effects over a number of generations, rather than mortality (Stauber 1995).

A major disadvantage of algal bioassays is that the response to environmental chemicals can range from highly sensitive to insensitive, depending on the species chosen. Clones of a species may also differ in their sensitivities, depending on the site of collection, so uniform clonal cultures must therefore be maintained in the laboratory for routine testing (Walsh 1993). Bioassays are usually carried out on single species, whereas species co-exist in natural systems (Stauber 1995), and laboratory tests are not necessarily predictive of field effects, due to the complexity of natural systems. Algal tests are often designed for single toxicants, whereas in reality contaminant mixtures are often encountered. Synergistic and antagonistic effects will not be revealed in single toxicant testing. Many of these limitations are equally applicable to other species used for toxicity testing (van Dam & Chapman 2001).

1.4.1 *Nitzschia closterium* bioassay

A sensitive bioassay for copper toxicity, using the estuarine unicellular diatom *Nitzschia closterium*, has been developed by CSIRO Centre for Advanced Analytical Chemistry (CAAC). The bioassay described in this report is a slightly modified version of the procedure reported in Stauber et al (1994) and Stauber et al (1996). The test uses *Nitzschia* cells in the exponential growth phase that have been cultured axenically in a special nutrient media in

which the trace element concentrations have been halved. Growth inhibition is used as an endpoint, after a 72 hour exposure to the test solution. Uniform clonal cultures of *Nitzschia closterium* are available from CSIRO Division of Marine Research (Tasmania) and CAAC.

2 Materials and methods

2.1 Collection of water samples

Macquarie Harbour is located in central western Tasmania, between 145º15'E and 145º 7'E, and 42º 1'S and 42º 55'S. The 'natural' water quality of the King and Gordon River catchments is typical of other west coast rivers, containing high concentrations of DOC (5 mg/kg) and humic substances that behave conservatively on mixing with seawater (Koehnken 1996, Eriksen et al 2001). A detailed description of the catchment hydrology, geology and chemistry of the study site is available in Koehnken (1996).

Samples for toxicity testing were collected in 1 L or 5 L LDPE sample bottles. New bottles were pre-soaked with a 0.1% Triton-X 100 (Sigma Chemical Co) solution for one week, and then rinsed copiously with deionised water to remove any traces of surfactant, before being placed in a 10% HCl bath for a further week. Sample bottles were then rinsed 5 times with deionised water, and left to dry. Bottles were capped tightly and bagged until required. All cleaning preparations were perfomed in a Class-100 clean room.

Salinity, temperature and pH profiles of the water column at the sample site were collected by deploying a Hydrolab Data Sonde 3 multiprobe from the boat and recording the data on a Hydrolab Surveyor 3 datalogger. This allowed the depth at which samples of a specific salinity were to be collected to be determined.

Two types of water sampling devices were used to collect field samples. Discrete water samples were collected using a Niskin bottle manufactured by the CSIRO Division of Marine Research (Hobart, Tasmania). The Niskin bottle allows 5 L samples to be taken at any depth, and is ideal for sampling when vertical gradients (eg salinity) are not too sharp. The Niskin bottle was attached to a polypropylene rope and triggered at the appropriate depth by a weight attached to the rope.

For very sharp vertical salinity gradients, a battery powered peristaltic pump was used to pump water onboard from a length of weighted silicon tubing let over the side to a predetermined depth. The tube inlet was modified to draw water over a very narrow vertical gradient, thus allowing very sharp salinity gradients to be sampled.

Samples were collected from a number of sites across Macquarie Harbour (fig 2.1). Sampling strategy was designed to capture inputs from the King River, which historically has carried mine waste (tailings, smelter slag and acid mine drainage) and topsoil into the harbour (Stations 3, 8, 9, 12), but is currently limited to AMD. Samples were collected at increasing distance from the point source, towards the Gordon River at the southern end of the harbour (Stations 27, 34, 35). Samples were also collected near the fish farms on the western shore (Stations 14, 15) and near the entrance to the harbour (Station 18).

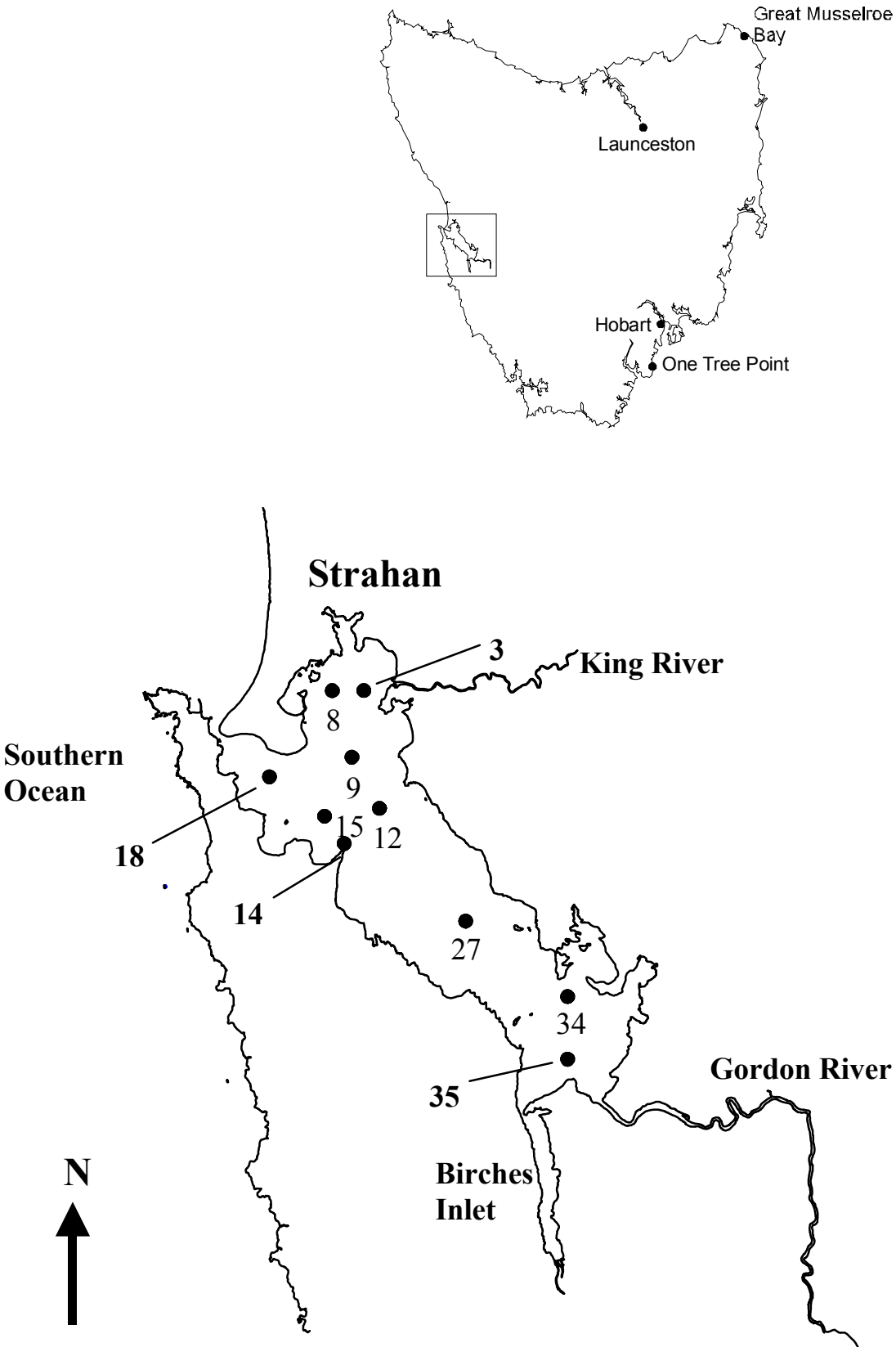

Figure 2.1 Collection sites for test organisms and sampling sites at Macquarie Harbour, Tasmania

2.2 Chemical methods

2.2.1 Total copper

Total copper measurements were determined by GFAAS (Mackey 1983). Filtered samples (0.45 μm) were acidified to pH 2 using high purity hydrochloric acid at the ratio of 1 mL acid/litre sample. The method uses APDC/DDDC (ammonium pyrrolidenedithiocarbamate / diethylammonium diethyldithiocarbamate) to extract the metals at pH 4. Metals are then back-extracted into high purity Milli-Q water at pH 2, resulting in a 50-fold pre-concentration factor. This level of sensitivity is required to measure background levels of copper in the dilution water and uncontaminated samples. Results are expressed as μg/L in the original sample. Stock solutions of copper to be used in all bioassays were analysed for total copper prior to use.

2.2.2 ASV-labile copper

ASV-labile copper measurements were made using a PDV-2000 portable analyser, in accordance with the method described in Koehnken (1996). A mercury film was plated onto the glassy carbon electrode, after polishing with alumina powder. Five mL of filtered (0.45 μm) sample was purged with nitrogen to remove oxygen, which may interfere with the analysis. Five mL of heavy metal electrolyte was added, and the sample analysed according to the manufacturer's recommended conditions. Additional measurements were made using a Metrohm 646VA Processor with hanging mercury drop. Samples were analysed under clean room conditions to prevent cross-contamination. Results are expressed as μg/L.

2.2.3 Free copper

Ion Selective Electrode (ISE) measurements were made in accordance with the protocols described in Eriksen et al (1999), on filtered (0.45 μm) samples at natural pH. An Orion copper (II) ISE was incorporated into a continuous flow analyser (CFA), in order to eliminate the contamination of samples by the electrode itself. The electrode was calibrated using metal ion buffers prepared with ethylenediamine (De Marco 1994). Standards in the range pCu_{free}14–9 (where $pCu_{free} = -\log[Cu^{2+}]$) were prepared by varying the pH of the buffer. Kinetic suppression of the chloride interference for buffers where $pCu_{free} > 9$ means that the ISE can be used for routine analyses in seawater. Lower pCu_{free} values (ie higher free copper concentrations) cannot be measured without invoking the chloride interference which has traditionally meant the ISE is overlooked for free copper measurements in saline waters.

pCu_{free} was calculated in MINTEQA2 (Allison et al 1996), using the stability constants reported in De Marco (1994). An extrapolation technique was used to process the data, to reduce the time required for sample analysis (Eriksen et al 1999). Filtered samples (0.45 μm) were stored at 4°C in the dark, and analysed as soon as practicable after collection to minimise the effect of speciation changes. As $pCu_{free} = -\log[Cu^{2+}]$, decreasing values on the pCu scale reflect increasing free copper concentrations. Since the response of the ISE is logarithmic, it should be noted this can disguise significant changes in the free copper concentrations.

The electrodes were regularly polished with 0.3 and 0.05 μm alumina powder to ensure a reproducible membrane surface that had a rapid response. The electrode was conditioned with clean filtered seawater until a stable potential was recorded (stability criterion 0.2 mV/min). Calibration with a series of pCu buffers (usually 5) was performed before each set of samples were analysed. All calibration slopes were in the range 27.7–29.4 mV/decade.

2.2.4 pH, salinity and temperature

Laboratory measurements of pH were made using an Orion Precision pH meter and Ross pH electrode. Salinity and temperature were measured using a WTW Microprocessor Conductivity Meter, Model LF196.

2.2.5 Nutrients

Nitrate plus nitrite and orthophosphate were measured using an Alpkem Auto-Analyser, and colourimetric methodologies (Eriksen 1997). Filtered samples (0.45 μm) were snap frozen until analysis.

2.3 Bioassays and culture of test organisms

A summary of all test conditions is given in table 2.1.

Table 2.1 Summary of toxicity test conditions

Test condition	*Nitzschia closterium*	*Artemia salina*	*Brachionus plicatilus*	*Heliocidaris erythrogramma*
Test type	Static	Static	Static	Static
Temperature	21 ± 1°C	20°C	25°C	20 ± 1°C
Light quality	Daylight fluorescent lighting	24 h dark	24 h dark	NA
Light intensity	14 000 lux	NA	NA	NA
Photoperiod	12 h light: 12 h dark	24 h dark	24 h dark	NA
Test chamber size	200 mL	5 mL	5 mL	10 mL
Test solution volume	100 mL	1 mL	1 mL	5 mL (5.1 mL after sperm addition & 6.1 mL after egg addition)
Renewal of test solutions	None	None	None	None
Age of test organism	5–6 days	48 h	0–3 days	Gametes
Initial density in test chambers	2–4 x 10^4 cells/mL	10/mL	5/mL	0.1 mL sperm + 1.0 mL eggs
Number of replicates	2–3	5	5	4
Shaking rate	Once daily by hand	NA	NA	NA
Dilution water	Natural < 0.45 μm filtered seawater	Natural < 0.45 μm filtered seawater	Natural < 0.45 μm filtered seawater	Natural < 0.45 μm filtered seawater
Effluent concentrations	Minimum of 5 and a control	4 + control	4 + control	Minimum of 5 + control + 4 references
Dilution factor	0.3 to 0.5	0.5	0.5	0.5
Test duration	72 h	24 h	24 h	1 h 20 min + prep
Endpoint	Growth (cell division)	Death	Death (no movement in 5 sec)	Fertilisation
Test acceptability	Control cell division rates 1.4 ± 0.2 doublings per day [a]	90% survival in controls	90% survival in controls	≥ 70% fertilisation in controls, and reference toxicant results within specified limits
Effluent volume required	< 500 mL	100 mL	< 100 mL	min 250 mL

[a] Salinity dependent, this data for salinity 35; NA Not applicable.

2.3.1 Sea urchin bioassay

Field collection and laboratory holding conditions

Heliocidaris erythrogramma is found around the entire Tasmanian coastline (Dartnall 1980) and along the east coast of Australia (Kobayashi 1980). They are typically found from 0.5 to 6 m depth, in rocky crevices and weedy substrates. Adults are up to 100 mm in diameter, with colour varying from light green to deep purple. Individuals cannot be sexed by external characteristics, and therefore must be induced to spawn or be dissected before sex can be determined. The reported spawning season in Australian mainland coastal waters is from December to March, with organisms passing through the mature, spent and regeneration phases 2 or 3 times (Williams & Anderson 1975). Eggs average 400 μm in diameter, and are positively buoyant. Sperm heads of *H. erythrogramma* are 10 μm in length. Laegdsgaard et al (1991) reported individuals less than 55 mm in diameter are usually not sexually mature.

The urchin *Heliocidaris erythrogramma* was collected on a number of occasions from early winter through to mid summer, as the availability and seasonality of eggs and sperm in Tasmanian waters was unknown. Urchins were collected by scuba divers from several sites around Tasmania. A collection permit is not required if small numbers of individuals are being collected. Initially urchins were collected from Great Musselroe Bay in King William National Park on Tasmania's north-east coast (fig 2.1). Great Musselroe Bay was selected because of its remoteness from human and industrial activities, and therefore the water quality would be expected to be good. Urchins were collected by levering with a flat blade knife taking care not to puncture the body (Simon & Laginestra 1997) nor tear off the tubercules. Since the degree and phase of reproductive activity was unknown, about 60 individuals were collected, and taken to the aquaculture facility at the University of Tasmania, Launceston. A premise was that one or two ripe males and females would be sufficient to conduct the test. The urchins were transported in 60 L fish bins, previously filled with ice and wet towels to prevent the urchins from overheating and prematurely spawning. Temperature was monitored regularly during transport. Urchins were transferred to 30 L glass aquaria filled with salinity 35 seawater. Tanks were aerated and 100% water exchanges made twice daily. Dissolved oxygen and temperature were regularly monitored. After artificial spawning, individuals were returned to the sea, or frozen if the condition of the animal was poor. Due to the long transport times between Great Musselroe Bay and the University, and the subsequent problems with premature spawning, an alternative sampling site was sought.

Further collections of urchins were made from sites near Hobart in mid summer. About 40 urchins were collected by scuba diving at One Tree Point, about 70 km south of Hobart. Populations of *H. erythrogramma* are healthy along this section of coastline (N Barrett, pers comm). Water temperature was 18°C, and salinity was 34.5 ppt. Urchins were transported to the aquaculture facility at the Tasmanian Aquaculture and Fisheries Institute (TAFI) in Taroona. The urchins were maintained in 200 L tanks with aeration and continuous exchange of salinity 35 seawater. Urchins were held for 24 hours before spawning was attempted. The final collection of urchins was made a fortnight later in early February. Forty individuals were collected by scuba diving from Taroona Beach, close to the holding facility at the Sea Fisheries Laboratories. It was not possible to access One Tree Point due to a road closure as a result of bushfires. The urchins were placed immediately in aerated seawater in 200 L tanks and left for 24 hours without feeding before spawning was attempted. The urchins were not fed after capture.

Sea urchin bioassay protocol

The urchin bioassay was adapted from the procedure developed by Simon and Laginestra (1997) for the urchin *Heliocidaris tuberculata*. The method is quite involved, so it is only briefly described here.

Individuals were spawned by injection of 2–4 mL of 0.5 M KCl into the peristomal cavity, and shaking or rotating the urchin gently for about 30 seconds. Eggs or sperm are released through the aboral surface, and males were kept as dry as possible to prevent premature activation of the sperm. *H. erythrogramma* gametes can be distinguished by colour: males produce white sperm, whilst females produce pink/orange eggs. Gamete quality is tested by activating the sperm with seawater. A very dilute suspension of eggs is added, and the mixture examined microscopically after about 3 minutes. Active swimming sperm and a high percentage of fertilised eggs (> 90%) indicate good gamete viability. Fertilised eggs are easily identified by a clear membrane, or halo, around the egg.

The appropriate sperm:egg ratio for toxicity testing was determined by measuring fertilisation success at a number of sperm densities. Egg density was held constant at 200 ± 10 eggs/0.1 mL. An ideal sperm:egg ratio is one that gives 85–90% fertilisation success in clean seawater.

The reference toxicant was copper sulphate and 2 types of controls were tested — filtered seawater and hypersaline brine adjusted to salinity 35. Copper concentrations tested were 15, 30, 60 and 120 μg/L (pCu_{total} 6.6, 6.3, 6.0 and 5.7) and 4 replicates were run at each concentration. The appropriate volume of sperm was added to each of the test tubes which were then covered and incubated at 20°C for 60 minutes. Eggs were then added to each tube, which was then covered and returned to the incubator for a further 20 minutes. The test was terminated by addition of buffered formalin. Fertilisation success was determined in the controls, to check that > 70% of the eggs had formed the membrane. Counting did not continue in the other treatments unless this criterion had been satisfied. Tubes were not pre-treated to reduce metal adsorption, although silanisation would be effective if it were shown to have no effect on the test sensitivity.

Samples are treated in the same way, except that the salinity of each sample must be adjusted to 35 using hypersaline brine. This means that for brackish waters, it is not possible to test undiluted samples. Hypersaline brine was prepared by freezing seawater in accordance with the method described by Simon and Laginestra (1997). Due to time constraints, only the fertilisation endpoint was measured in this study. Abnormal development is a common and ecologically relevant endpoint that could also be included in similar toxicity studies.

2.3.2 Brine shrimp bioassay protocol

Brine shrimp cysts were obtained from the Tasmanian Aquaculture and Fisheries Institute (TAFI). Premium brine shrimp eggs (supplied by Prime Artemia Incorporated, Utah, USA) were hatched by adding cysts to seawater, exposing to a light source for an hour and then incubating in darkness 48 hours before the test commenced. Good hatch rates can be obtained in salinities greater than 15 and pH range 7.5–8.5. After the incubation period, which included transfer to fresh medium after 24 hours, ten larvae were placed into each testing well. Copper concentrations tested were 0, 10, 20, 40 and 80 μg/L Cu (added as copper sulphate, pCu_{total} 6.8, 6.5, 6.2 and 5.9). Five replicates were used at each test concentration. The wells were covered and incubated in darkness at 25°C for 24 hours. After 24 hours the dead larvae were counted and percentage mortality determined in each treatment. Mortality in the controls should not exceed 10% for the test to be valid.

2.3.3 Rotifer bioassay protocol

Branchionus plicatilis is the main rotifer species used for estuarine toxicity testing (Widdows 1993). The test is an acute toxicity test using newly hatched animals (within 2 hours of cyst hatching) and the starting time is crucial for the success of the test. Five replicates were used at each test concentration. As a mixed culture of unknown age (0–3 days) was the only available source of the experimental animals, the results should be interpreted with caution. Five rotifers were transferred to each of the replicate wells; the wells were covered and incubated for 24 hours. After 24 hours, each of the wells was scored for the dead rotifers (no movement in 5 seconds) and the percentage mortality was calculated. Mortality in the controls should not exceed 10% for the test to be valid.

2.3.4 Algal bioassay protocol

Algal culture

The algal strain *Nitzschia closterium* (Ehrenberg) W Smith (Strain CS 5) was obtained from the CSIRO Division of Marine Research Microalgal Culture Collection, Hobart, Tasmania. The culture was maintained axenically using a low-metal culture media (table 2.2,) at 20°C, on a 12 h light : 12 h dark cycle using fluorescent daylight bulbs, as per Stauber et al (1994). A summary of the culture media is included in table 2.2.

Table 2.2 Summary of modified f-media for culturing *Nitzschia closterium*

Nutrient solution	Test concentration (mg/L seawater medium)
1. $NaNO_3$	150
2. $NaH_2PO_4.2H_2O$	10
3. $NaSiO_3.5H_20$	25
4. Ferric citrate/citric acid	
$C_6H_5O_7Fe.5H_20$ +	4.5
$C_6H_8O_7.H_2O$	4.5
5. Trace Elements	
$CuSO_4.5H_2O$	0.009
$ZnSO_4.7H_2O$	0.022
$CoCl_2.4H_2O$	0.010
$MnCl_2.4H_2O$	0.180
$NaMoO_4.2H_2O$	0.013
6. Vitamins	
Biotin	0.001
Vitamin B12	0.001
Thiamine	0.2

Cultures were transferred into new media weekly so that the cells in the exponential growth phase were continually available. Using sterile technique, 1 mL of inoculum was transferred from the culture to a flask containing 75 mL of autoclaved media and incubated using the above conditions until the culture was 5 days old and cells were in the exponential growth phase. The source cultures are maintained at CSIRO in a standard f-media, so the purchased culture must be taken through at least 3 inoculations into the modified media described in Stauber et al (1994) before the algae can be used for toxicity testing (Leroi, pers comm). This

ensures that the algae are acclimated to the new nutrient/metal concentrations before tests begin. After inoculation into the low-metal media, daily cell counts were performed to confirm that a 5 day old culture would provide sufficient cell density for the bioassay.

Algal bioassay

Nitzschia closterium cells were inoculated into flasks containing 100 mL of filtered seawater collected from Maria Island, Tasmania. The algal cells were taken through a washing procedure prior to inoculation to remove all traces of the culture medium as this has been shown to affect the degree of copper toxicity (Stauber & Florence 1989). Flasks were spiked with 1.0 mL of 1.3 mM NaH_2PO_4 and 1.0 mL of 26 mM $NaNO_3$ to give final nutrient concentrations of 130 μM phosphate and 260 μM nitrate. Flasks had been previously coated with Coatasil (Ajax) to reduce loss of added copper to the glass. Silanised flasks were acid washed and rinsed copiously with high purity MilliQ water prior to use in bioassays.

For the ionic copper calibrations, flasks were spiked with 0, 2.5, 5.0, 10.0, 20.0, 40.0 or 80.0 μg/L of copper using copper sulphate with two or three replicates at each test concentration. Total copper in the flasks was corrected for the initial concentrations of copper in the Maria Island seawater. Initial concentrations ranged from 0.09 to 0.11 μg/L. Two salinities, 20 and 30, were tested. Flasks were mixed thoroughly and placed in culture cabinets preset to the required temperature and light regimes. Initial cell density was between 2 and 4 $x10^4$ cells/mL. Cell density was determined daily in each flask.

Samples from a number of sites around Macquarie Harbour (salinity = 20) were screened for toxicity using undiluted sample. Conditions were identical to those described for the copper addition experiments. Nutrients were determined in the samples and an adjustment made to the amount of nutrient stock added so that the final concentration was the same as the control water.

Cell counts

Manual counts of cell density were performed using an Improved Neubauer Hemacytometer, with a Leitz Wetzlar compound microscope, at 100 x magnification. Each flask was sub-sampled axenically on day 1, 2 and 3 of the test. Cell densities on day 0 were assumed to be identical in all flasks.

Flasks treated with Coatasil were found to be 'sticky' with the algae difficult to remove from the bottom of the silanised flasks. This significantly affected cell density determinations because the flask contents were not homogeneous, even after extended swirling. To overcome this problem, small acid-washed, Teflon-coated magnetic stirrers were placed in each flask and the flasks gently moved over a magnetic stirrer on low speed. This lifted the algae from the bottom of the flask and stirred the contents of the flask at the same time. This process did not appear to affect the algae (ie no physical damage apparent), and the stirrer bars did not present a contamination source. Only 1 mL of sample was required for microscope counts. It was not necessary to use a tissue grinder to break up clumps of algae prior to counting. If cell counts could not be performed immediately, samples were preserved with a buffered formalin solution.

At the conclusion of the test, growth rates were calculated by plotting cell density against time (hours) and determining the slope of the line for each flask by regression analysis as per Stauber et al (1994). The slope of the line is equivalent to the growth rate per hour (μ) for each treatment. Daily doubling times are then calculated from 24 x μ x 3.32. Initial periods of lag growth were noted, but not taken into account in determining the average slope for each treatment. Acceptable control growth rates vary with the test salinity, and details are provided in the discussion.

3 Results and discussion

3.1 Brine shrimp bioassay

The brine shrimp assay proved to be insufficiently sensitive to copper, over the concentration range tested. There were no mortalities in the 10, 20 or 40 μg/L treatments, and a single mortality in one of the replicates at 80 μg/L. There was also a single mortality in one of the control replicates, giving a result of 10% mortality. Thus the test was valid, but obviously unsuitable as a sensitive test for copper toxicity, since the LOEC determined for this species was > 80 μg/L total nominal copper. No further tests were conducted with *Artemia salina*.

3.2 Rotifer bioassay

The rotifer bioassay also proved to be unsatisfactory for further study. Repeated mortalities (> 10%) in the controls meant that the bioassays were not valid. It was assumed that the difficulties with the bioassay were a result of using rotifers from a continuous culture system, rather than newly hatched organisms of known age. Given the reported insensitivity of the species to copper (24 hr LC_{50} of 130 μg/L), lack of ecological significance, and our inability to obtain cysts, further tests were not conducted.

3.3 Sea urchin bioassay

The sea urchin fertilisation bioassay was conducted in accordance with the protocols described for *Heliocidaris tuberculata* (Simon & Laginestra 1997). An ionic copper calibration was performed to determine if local urchins had sufficient sensitivity to copper to warrant further investigation. The protocol for *H. tuberculata* used 15–160 μg/L copper, plus controls, but the higher concentrations (> 60 μg/L) exceed the solubility of copper in seawater (determined on low DOC, open-ocean water, D Mackey, pers comm). For the purpose of this study, a slightly reduced range of test concentrations were used, but it should be noted that the highest test concentration result should be treated with caution.

Clean seawater spiked with copper (0, 15, 30, 60, 120 μg/L Cu using $CuSO_4$) was used to test sperm viability. Four replicates were prepared at each test concentration. Sperm were exposed to spiked seawater for one hour and then eggs were added for a 20 minute period.

Test acceptability is defined as greater than 70% fertilisation in filtered seawater controls, and this test yielded 87.5% fertilisation (fig 3.1). There was a linear relationship between total copper concentration and percentage of eggs fertilised by exposed sperm ($y = -0.43 x + 85.34$, $R^2 = 0.88$, $p < 0.01$) with a LOEC of 30 μg/L total nominal copper, and an EC_{50} of 71 μg/L. However, the test was not as sensitive as required and was generally a time-consuming assay to run.

Kobayashi (1980) reported that *H. erythrogramma* was suitable for short-term testing (fertilisation success and larval development) as the organism has rapid development from fertilisation to metamorphosis, with larvae settling within 4 to 5 days. The urchin test was selected because it potentially offered a sensitive bioassay using organisms that were easy to collect and maintain in laboratory conditions. However, significant problems were encountered obtaining sufficient eggs and sperm to conduct the tests on a regular basis. It was found that the maturation of gonads of *H. erythrogramma* is limited to a very short season and varies significantly between collection sites around Tasmania. Kobayashi (1980) collected urchins from the more temperate waters off Sydney, and the urchins reportedly have a much longer spawning season.

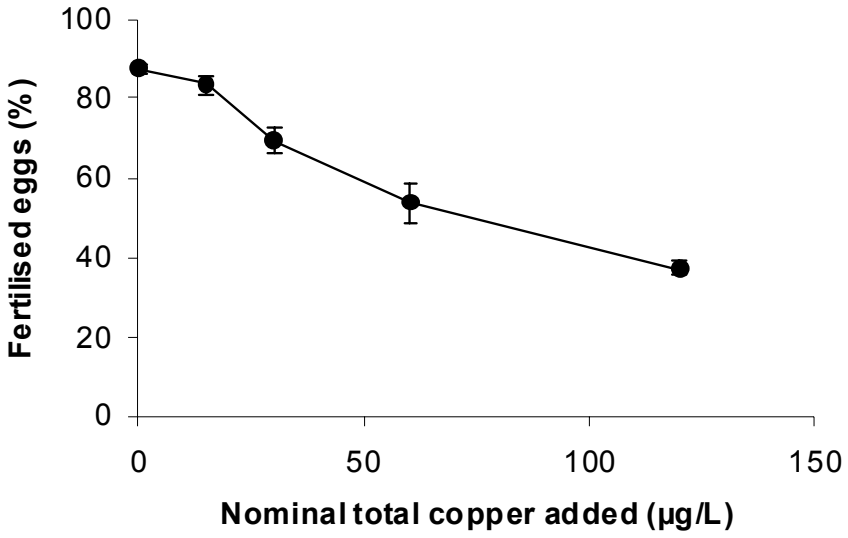

Figure 3.1 Fertilisation success of *Heliocidaris erythrogramma* after sperm exposure to copper for 1 hour (Mean ± SE)

On the first sampling occasion, only females were successfully spawned and several individuals produced small quantities of pink or orange eggs. Microscopic inspection revealed that the eggs were very irregular in size, suggesting that the gonads had not yet matured. No males were induced to spawn on this occasion. The condition of the urchins deteriorated markedly after 2 days and they were returned to the sea.

For urchins collected in mid-summer, there was no improvement in spawning success. On the first day, 3 females produced a very small number of eggs, insufficient for the test requirements. No males were induced to spawn. All injected individuals were placed in a separate 200 L tank. A second attempt was made the following day; this time 5 males produced significant quantities of sperm but again insufficient eggs were collected to warrant continuing with the test. The condition of the urchins deteriorated rapidly after spawning and the majority were returned to the sea.

Urchins collected from Taroona in February were the most responsive to artificial spawning methods, suggesting strongly that the spawning season in Tasmanian waters is much shorter than for the warmer waters off Sydney. A small number of males and females spawned, with eggs again being most difficult to collect. The bioassay was conducted using the method of Simon and Laginestra (1997) but with a lower density stock egg solution. This resulted in all eggs being counted in all test tubes and percentage fertilisation success being calculated as a function of the total number of eggs used in each tube. This differed from the published protocol where only the first 100 eggs are counted.

Eggs may also be collected by dissection but it is difficult to separate the individual eggs from the egg mass and the method does not necessarily produce mature eggs suitable for use in a routine assay. Williams and Anderson (1975) reported artificial fertilisation of *Heliocidaris erythrogramma* by shaking pieces of gonad into beakers to release the gametes. In this study, an attempt to release gametes from the gonad mass by dissection was found to be time consuming and largely unsuccessful.

Insufficient knowledge about the intricacies of natural spawning cycles of local populations of *Heliocidaris erythrogramma* meant that it was unsatisfactory to rely on regular collection of individuals in the field as a source of viable gametes. For future work, it would be worthwhile setting up a permanent holding facility, at least over the summer months, where urchins could

be maintained under strictly controlled conditions. Alternatively, regular field trips to a number of local sites would be beneficial. Inducing urchins to spawn in the field on these field trips would allow tests to be conducted on a more opportunistic basis and would increase our knowledge of the urchins spawning behavior. However, it has been shown that repeated collection of urchins from the same sites for toxicity testing can lead to localised population decline (Byrne & Laginestra 1999). A further option would be the collection and use of frozen embryos and gametes which would prove beneficial if the local species do indeed have a very limited spawning cycle or are in low supply. Investigation into suitable conditions for cryopreservation would be necessary.

In summary, the short summer spawning period means that animals can only be collected for a limited part of the year. It was beyond the scope of this study to attempt to maintain animals in spawning condition for long periods of time. As a result of the unsatisfactory results with the invertebrate tests, all further studies were carried out with the diatom *Nitzschia closterium*. The algal bioassay was found to be the most robust test and, most importantly, had the required sensitivity for subsequent investigations with environmental samples.

3.4 Algal bioassay

3.4.1 Algal culture and inoculation

Cell counts performed on a series of *Nitzschia* cultures previously acclimated with the low-metal culture media showed that after 5 days, cell densities had reached 9 x10^5 cells/mL (fig 3.2). Cell densities of 10^6 were recorded after day 6 but cells were moving out of the log phase growth and into the stationary phase, which would result in a significant lag in the growth of the cells in the inoculated samples. Conversely, at day 4, when samples are at the beginning of the exponential growth phase, cell densities are too low to use without significant dilution of the sample to be tested, or culturing large quantities and pre-concentrating them. Inoculum cell densities are therefore a compromise between culture age and growth phase.

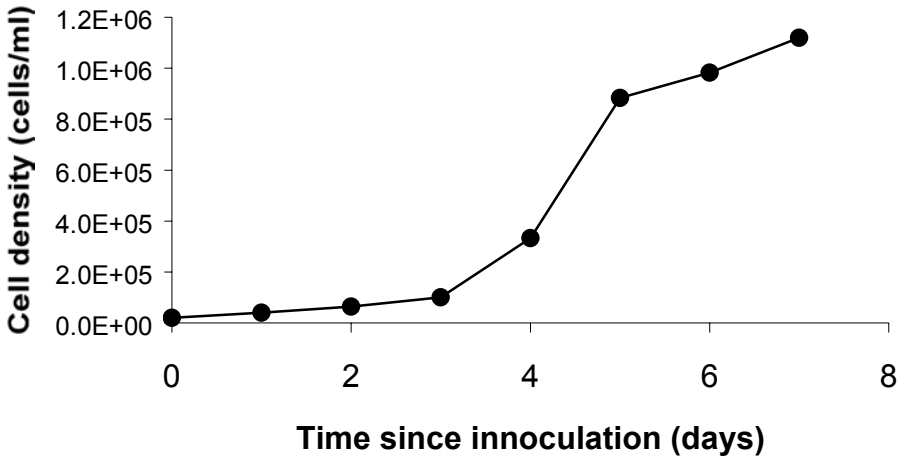

Figure 3.2 Growth curve for *Nitzschia closterium* (CS5) in modified f-media

3.4.2 Calculation of growth rates at salinities not previously tested

Salinity has a significant effect on the growth rates of *Nitzschia closterium*, so in order to calculate the effect of sample toxicity on *Nitzschia*, the sample salinity must be taken into account. Stauber et al (1994, 1996) have reported variations in doubling rates per day at a

number of salinities so that test acceptability can be defined for a range of sample types (fig 3.3). Doubling rate is linearly related to salinity ($y = 0.03x + 0.40$, $R^2 = 0.96$, $p < 0.01$) and these data were used to predict the doubling rate of *Nitzschia* under identical culture conditions at salinity 30. The predicted doubling rate was 1.3 doublings per day, and the observed doubling rate was 1.3 doublings per day. Therefore, 1.3 doublings per day was used as the criteria for test acceptability of toxicity tests conducted for this study.

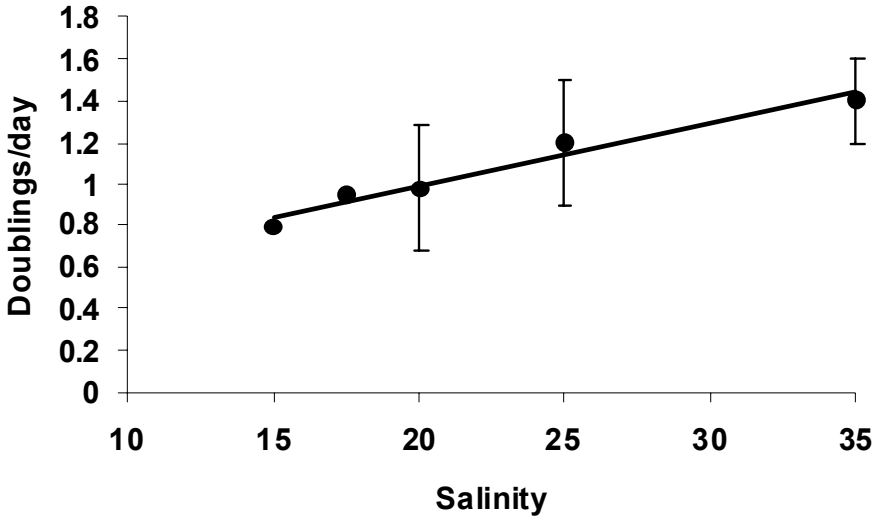

Figure 3.3 Variation in doubling rate per day of *Nitzschia closterium* with salinity (data from Stauber et al 1994 & 1996). Error bars (ranges) included where data provided.

3.5 Ionic copper calibration at salinity 20

The effect of copper on the growth rate of *Nitzschia closterium* at salinity 20 was tested by adding copper (as $CuSO_4$) to a series of flasks containing clean seawater and nutrients. The algal inoculum was rinsed three times to remove any traces of culture medium, according to the method of Stauber (1994) and Stauber et al (1996). Raw data are shown in figure 3.4.

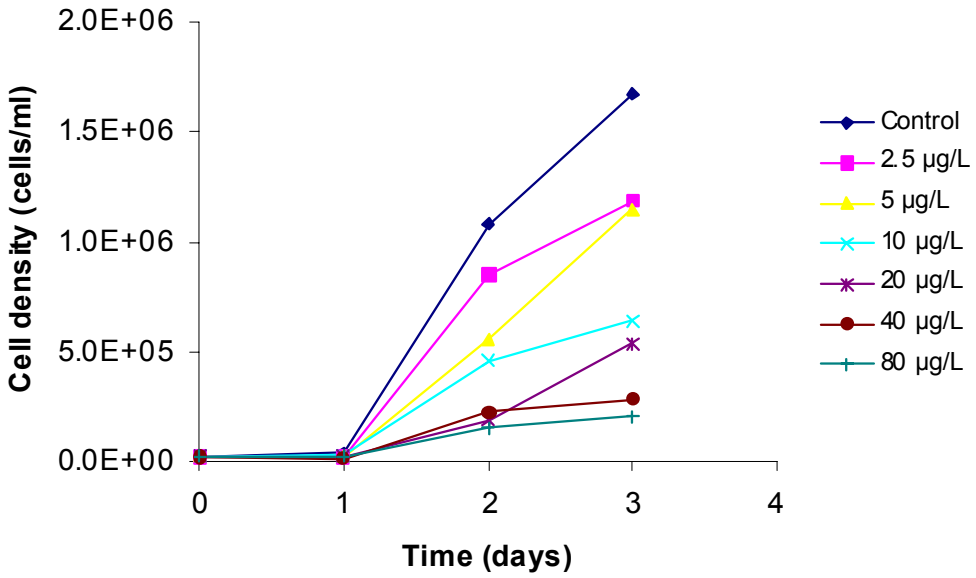

Figure 3.4 Effect of exposure to copper on growth of *Nitzschia closterium* at salinity 20

In all test flasks there was a lag on day 1 of the test, indicating that the cells used to inoculate the sample were approaching the stationary phase. Tests for acceptability of the control showed that the bioassay was acceptable with 1.2 doublings/day (Stauber et al 1996).

Nominal total copper concentrations were 2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 µg/L.

3.6 Copper speciation and toxicity

The effect of copper on the growth of *Nitzschia* was plotted as a function of ASV-labile copper, total nominal copper and free copper on Day 0 (figs 3.5, 3.6, 3.7). Growth rates of the algae for the various concentration treatments are expressed as a percentage of the control. Filtered samples were collected at the conclusion of the 72 hour assay and re-analysed to determine if there were significant losses of copper from solution. After 3 days, there was less than 50% of the total nominal copper concentration left in solution, despite silanisation of the flasks. The most likely cause of the reduction in total copper concentration is adsorption of copper onto the cells themselves, and/or lossess from solution by precipitation or other mechanisms. This highlights the problem of using nominal metal concentrations in toxicity tests, but may be overcome by the direct measurement of the free metal ion (by ISE). Alternatively, it is possible to quantify non-biological losses of total copper by conducting an identical series of experiments, without algae, and analysing for total copper at the beginning and end of the test (J Stauber, pers comm). Studies of copper losses to glass flasks have shown that commonly 10%, and up to 40% of copper may be lost by adsorption alone (Batley et al 1999).

Significantly, Florence et al (1983) found that adding copper sulphate (5–50 µg/L) to flasks containing 10^7 cells/L of *N. closterium*, resulted in 20–45% of the copper being adsorbed to the cells, suggesting this is an important factor for consideration when using total or nominal metal concentrations. pH was tested and found not to be significantly different from the initial test value (8.03 ± 0.02).

Similar trends were found for toxicity as a function of both labile and total copper. The LOEC for total nominal copper was determined to be 5 µg/L. There was a 'plateau' in the sensitivity at the lower end of the copper range, between 2.5 and 5 µg/L Cu. This plateau was not evident at 48 hrs (see fig 3.4). At concentrations ≥ 5 µg/L the algae were more sensitive, with a concentration dependent relationship. The 72 hr EC_{50} value was in agreement with the value of 18 µg/L determined by Stauber et al (1996). ASV-labile concentrations were between 56 and 80% of the nominal total copper concentrations.

Toxicity as a function of free copper measured by ISE on day 0 is shown in figure 3.7. Growth rates decreased from 100% in the controls to less than 8% in the highest copper treatment (80 µg/L nominal total copper, pCu_{free} = 8.2). Free copper represented a very small proportion of the ASV-labile copper in solution, and there was a strong correlation between toxicity and $pCu_{free(ISE)}$ ($R^2 = 0.94$, $p < 0.01$) over the whole concentration range tested. The pCu_{free} values measured on day 0 ranged from 11.8 (controls) to 8.20. The LOEC value determined for this test was pCu_{free} = 10.9. Very low free copper concentrations are likely to be due to complexation (both organic and inorganic), as well as wall effects, losses during filtration, precipitation from solution, and interactions with algae and colloidal substances present in solution.

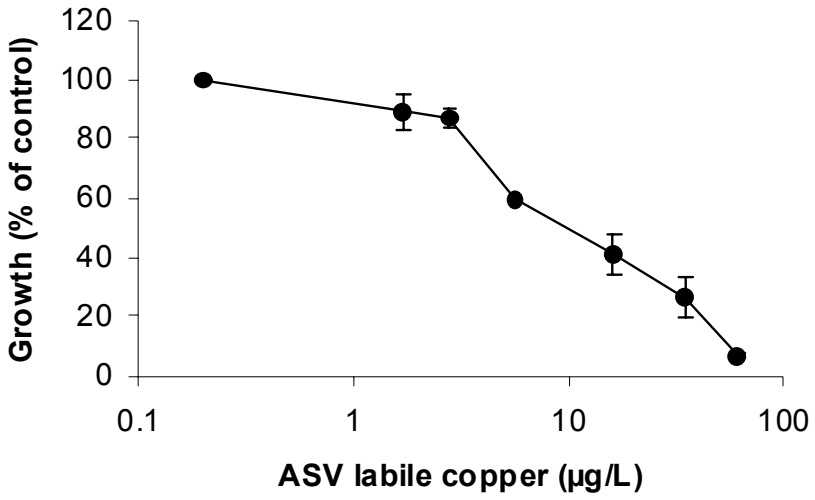

Figure 3.5 Growth of *Nitzschia closterium* (mean ± SE) vs ASV labile copper, at salinity 20

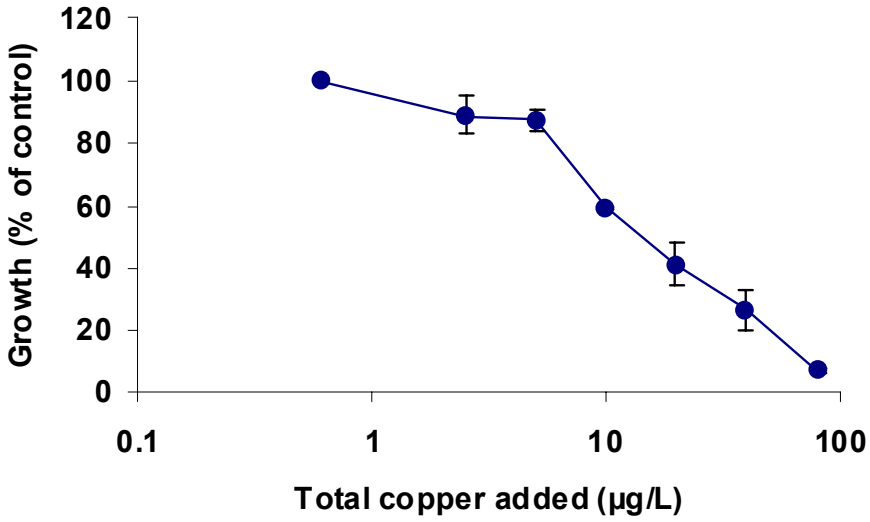

Figure 3.6 Growth *of Nitzschia closterium* (mean ± SE) vs total copper added, at salinity 20

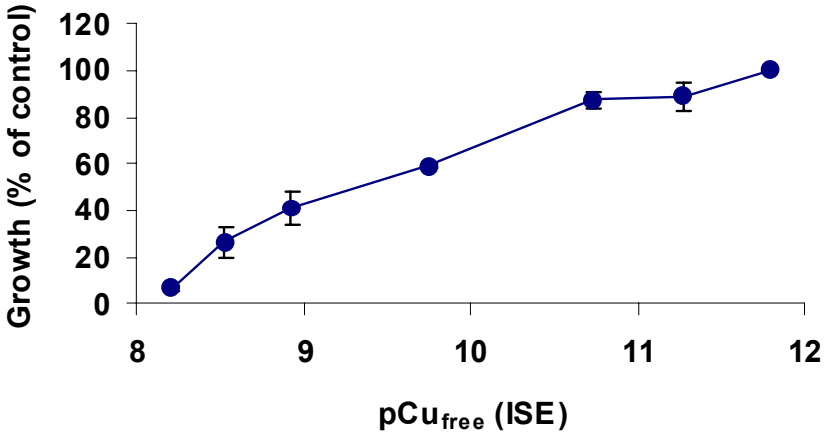

Figure 3.7 Growth inhibition of *Nitzschia closterium* (mean ± SE) vs free (ISE) copper, at salinity 20

3.7 Ionic copper calibration at salinity 30

Results for the ionic copper calibration at salinity 30 are shown in figures 3.8 and 3.9. In this test, no lag was observed on day 1 of the test, indicating that the stock culture was in the exponential phase when the inoculum was prepared. However, the cell yield was lower in salinity 30 water than in salinity 20 (5 x 10^5 cells/ml compared with 1 x 10^6 cells/ml). The reason for this difference is unknown. Since ASV labile results at salinity 20 did not differ significantly from total copper, only total copper and free copper were analysed for salinity 30 tests. Growth rate in the controls was 1.3 doublings per day.

At salinity 30, the growth of the algae decreased as the concentration of total copper in solution increased (fig 3.9). The response to free copper, as determined by ISE, was similar to the test at salinity 20 — ie as free copper increased, population growth decreases (fig 3.10).

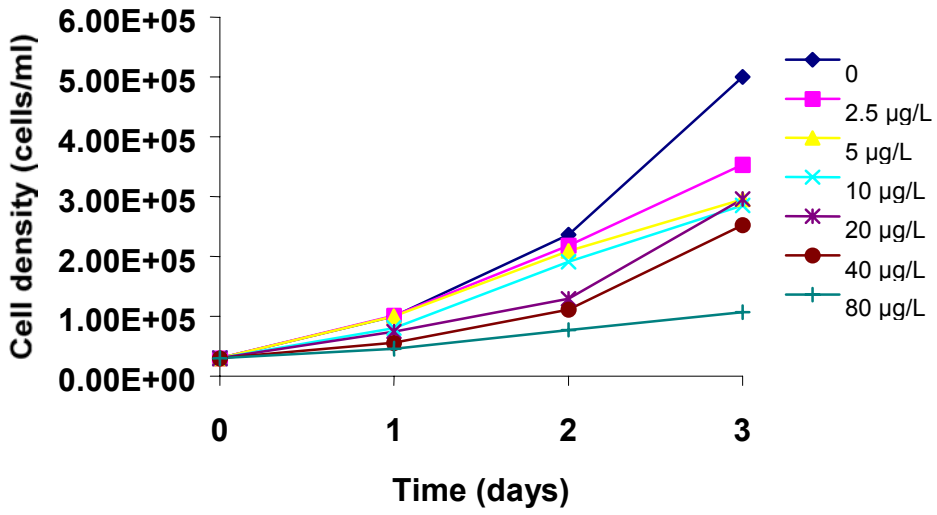

Figure 3.8 Effect of exposure to copper on growth of *Nitzschia closterium* at salinity 30

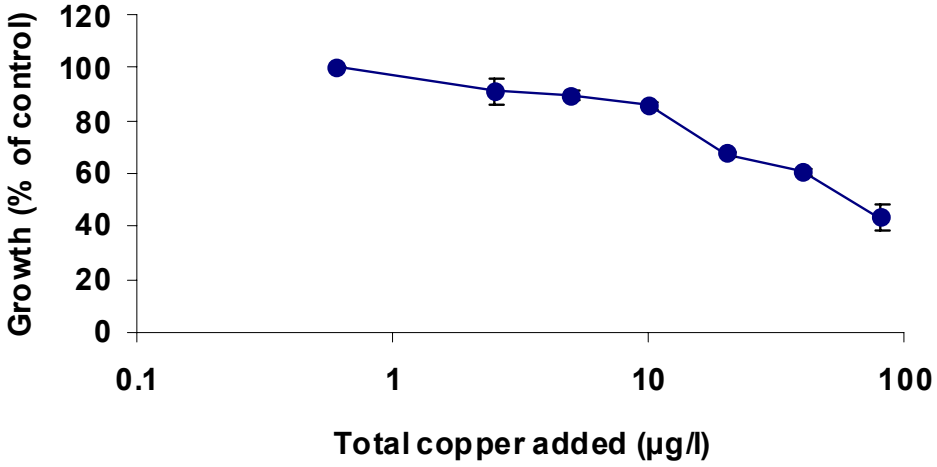

Figure 3.9 Growth *of Nitzschia closterium* (mean ± SE) vs total copper added, at salinity 30

The range of pCu_{free} values measured in the spiked samples at salinity 30 (pCu 11.9–8.5) is slightly offset from the salinity 20 trial. There was a slightly weaker correlation between ISE response and growth inhibition ($R^2 = 0.91$, $p < 0.01$) and the LOEC determined for this test was pCu_{free} =11.3. The same plateau described in the salinity 20 tests was detected at low concentrations of free copper in the salinity 30 trial.

The toxicity of copper to *Nitzschia* was less at the higher salinity, with 80 µg/L nominal copper reducing growth rates to 40% of the controls, compared with less than 10% in the salinity 20 trial. The reason for the difference in sensitivity is not known, but Florence (1989) has suggested that algae are less stressed by toxicants at higher salinity. This is feasible considering a similar range of pCu_{free} values were measured at both salinities tested. Stauber et al (1996), however, found the 72 hr EC_{50} value for *N. closterium* (total nominal copper) was lower at salinity 35 than salinity 20 — ie copper was more toxic at higher salinity.

In this study, the concentration of pCu_{free} measured in clean seawater controls (pCu_{free} 11.8 at S = 20, 11.9 at S = 30) seems reasonable in comparison with other studies of pCu_{free} in estuarine and coastal marine waters. Sunda and Ferguson (1983) estimated ambient pCu_{free} to be 11.5 in water from the coast of Peru and 11.3 and 11.5 for samples from the Northern Gulf of Mexico, and was reported as between 11.0 to 12.3 for other sites (Sunda & Lewis 1978). Zirino et al (1998) measured pCu_{free} values of 11.6 for waters outside the mouth of San Diego Bay.

The range of pCu values over which toxicity is observed in this study is also consistent with literature reports. Sunda and Guillard (1976) reported toxicity in the marine diatom *Thalassiosira pseudonana* over the range pCu_{free} 11.2–8.2. In their study, free copper was manipulated by the use of metal ion buffers in the bioassay solution. Using an NTA-cupric buffer system, Brand et al (1986) found reduced rates of growth in seven species of marine diatoms at pCu_{free} 10.5–11.0. Additionally they reported reduced rates of reproduction in two species of dinoflagellates at $pCu_{free} < 12$. The dinoflagellate *Gonyaulax tamarensis* was found to be sensitive to copper over the range pCu_{free} 13.7–9 (Anderson & Morel 1978) in artificial seawater. It should be noted that buffer systems were not employed in this study to regulate the free copper concentration, and natural, not synthetic seawater was employed for all tests.

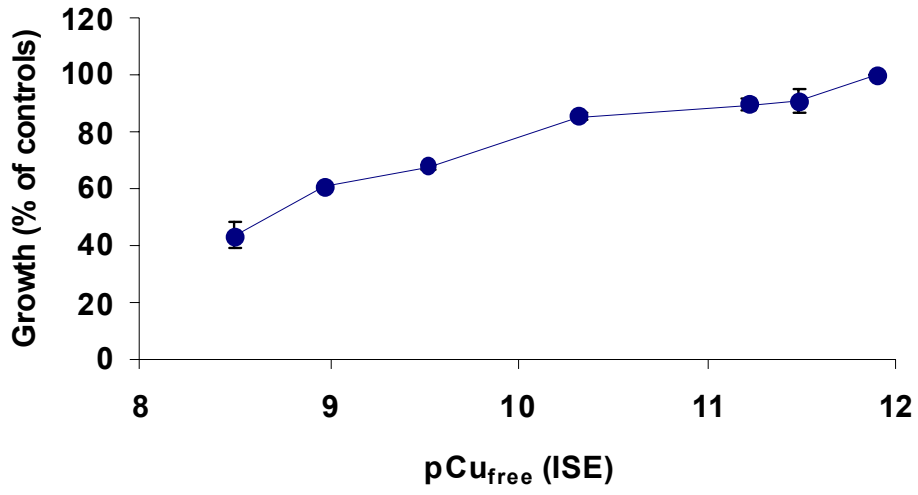

Figure 3.10 Growth of *Nitzschia closterium* (mean ± SE) vs free (ISE) copper at salinity 30

3.8 Macquarie Harbour samples

pCu_{free} was measured in a number of samples collected from the salinity 20 isohaline at stations 3, 8, 9, 12, 14, 15, 18, 27, 34 and 35. pCu_{free} values fell over a comparatively narrow range from 11.3 to 12. Total copper for these samples ranged between pCu 6.8 and 6.6 (10.63 and 17.06 μg/L), indicating that a very small proportion of copper was present as the free metal ion. Predicted toxicity, based on the ISE results in figure 3.7, was between 96 and 111% of growth in the controls, ie no toxicity was expected. Predicted toxicity was greatest at station 3, closest to the mouth of the King River (96% of control growth) and least at station 35, closest to the Gordon River (111% of control growth). Growth rates of *Nitzschia* in field samples from all sites varied between 87 and 107% of the controls, though neither growth enhancement or inhibition were statistically significant (Dunnetts test $\alpha = 0.05$). The fact that a very strong relationship exists between growth inhibition and ISE response in spiked seawater indicates that the ISE tracked changes in the free copper concentration in solution over almost 4 orders of magnitude. The ISE showed insufficient free copper was present to cause growth inhibition in the algae, and this is in agreement with earlier studies in Macquarie Harbour which also observed no toxicity to *Nitzschia* (Stauber et al 1996).

Predicted toxicity in samples from Macquarie Harbour using ASV-labile copper was much higher than that predicted using the ISE. ASV predicted growth inhibition of up to 50%, compared with salinity matched controls. ASV labile measurements over-estimated the toxicity of the samples, and this may have been due to the fact that the method measures free copper as well as labile copper complexes that dissociate or are directly reduced at the electrode surface. These labile copper complexes may include inorganic and weakly bound organic complexes (Eriksen et al 2001). The ASV results are in good agreement with earlier work by Stauber (Stauber et al 1996) which also showed that ASV labile copper was not a good indicator of the potential toxicity of Macquarie Harbour water samples.

4 Conclusion

The algal bioassay proved to be an excellent test to investigate the relationship between chemical estimates of toxicity (ie labile and free copper) and biological estimates of toxicity (ie growth inhibition). No significant problems were encountered with the bioassay, or the culture of the algae. A recommended amendment to the method would be to include a chemical series of flasks to enable an estimate of non-biological losses of copper, as discussed in section 3.6. In flasks containing algae, significant losses of copper occurred, although Florence et al (1983) have shown that up to 45% of copper in solution may be directly adsorbed onto *Nitzschia* cells. A mass balance for copper would confirm if that is the case in this study. This would involve total copper analysis of the filtered water, the filtrate (ie algal cells plus particulate matter), and acid leached copper (from the walls of the flask). Additionally, direct measurement of the free copper in solution with the ISE over the duration of the tests would be beneficial to determine the partitioning of copper added to the samples. Unfortunately it was not possible to carry out these tests due to time restraints.

Ionic copper additions to clean seawater were conducted to establish which of the analytical techniques showed most potential for application to the study of a contaminated estuary. There have been examples in the literature of good correlation between ASV-labile copper and toxicity in laboratory studies (Florence et al 1983). There have been fewer examples of how this relationship holds in the field. In Macquarie Harbour, recent studies have shown that ASV-labile copper was not a good indicator of toxicity. High concentrations of labile copper were measured, and toxicity was expected on the basis that the labile metal was bioavailable

(Stauber et al 1996). Growth inhibition tests conducted using *Nitzschia closterium* showed that the harbour waters were not toxic to algae. Similar results were found in this study. The concentration of labile copper measured in Macquarie Harbour was sufficient to expect significant (~50%) growth inhibition, based on the ionic copper calibration test.

The copper ISE response was found to correlate strongly with growth inhibition of *Nitzschia*, compared with salinity matched controls. The concentration of free copper measured was very much lower than the total or the ASV-labile copper in solution. Free copper in the controls was measured as pCu 11.9, a result comparable with literature concentrations in clean seawater. The range of pCu_{free} values over which a decrease in growth rate was observed (pCu 11.3–8.2) was also in good agreement with data reported in the literature for other species of algae. The results of this study suggest strongly that the ISE could be used for more detailed studies of copper toxicity. The major advantage of the ISE is that it can be used for the direct measurement of free copper in seawater, thereby avoiding many of the complications arising from artificially manipulating the free ion concentration, and the problem of metal partitioning in the test container.

The relative sensitivity of the tested species to copper was alga > sea urchin > rotifer > brine shrimp. Whilst the brine shrimp and rotifer tests were extremely simple and quick to run, the insensitivity of the species precludes their use for detailed studies of copper toxicity and speciation. The sea urchin bioassay is potentially a valuable test, if the difficulties in gamete collection can be overcome. Larval development may be a more sensitive and complimentary test to run in conjunction with the algal bioassays. Algal tests were found to be the most sensitive, easiest to run on a continual basis, and were not affected by seasonal constraints. Algae are relatively easy to culture continuously in the lab, and as primary producers, have been shown to be relevant ecologically due to their position at the base of the food chain (Stauber 1995).

Chemical analysis alone may not reflect the potential toxicity of polluted waters to bioassay organisms, depending upon the method of analysis chosen. However, if we have site specific knowledge of the form or isomer that is expected to be toxic, and given that there are no synergistic or antagonistic effects occurring, we can predict the relative toxicity of a sample. Maximum information is obtained by combining suitable chemical estimates of toxicity with relevant, sensitive bioassays.

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