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MOUNT LYELL REMEDIATION

Effects of exposure to sub-lethal levels of copper on growth and health of sea farmed rainbow trout

Barbara Nowak & Susan Duda

Mount Lyell Remediation Research and Demonstration Program

a Tasmanian and Commonwealth Government initiative

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Department *of* Environment and Land Management

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

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

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Executive summary

Macquarie Harbour is an almost entirely land-locked estuarine harbour with an excellent potential for fish farming and already has a number of established aquaculture sites. However, there is concern about the potential effects of pollution from the King River on the fish farming in Macquarie Harbour. The operation of the Mount Lyell Mine at Queenstown over the last 100 years resulted in significant pollution of the harbour with copper and to a lesser extent other toxic metals. Even though the new operator of the mine, Copper Mines of Tasmania Pty Ltd, has constructed a tailings dam, acid drainage from historic mining operations remains a major environmental concern, and there are elevated levels of copper in parts of the harbour, particularly around the mouth of the King River. In these areas, fish may be exposed to harmful levels of copper. Two species cultured in the harbour are rainbow trout and Atlantic salmon. Both species are hatched and reared in freshwater hatcheries for about a year before being moved to sea-cages where they grow to marketable size.

Copper is one of the most toxic heavy metals to fish. Though numerous studies have been carried out on the effects of copper on fish, most of them focused on the early life stages of fish in freshwater. Little is known about the potential effects of exposure to copper on salmonid fish during grow-out in sea-cages under the brackish water conditions that exist in Macquarie Harbour.

The aim of this project was to determine effects of copper on growth and health of rainbow trout in brackish water. A wide range of tests was chosen to establish the effects. The effects on growth were determined by measuring wet weight gain during the exposure time. Additionally, sensitive biochemical tests were carried out to find any growth effects which would not be obvious in weight changes after a few weeks. These tests included protein content in white muscle and the RNA:protein ratio in white muscle. The health of the fish was measured by testing effects on non-specific immune response (phagocytosis assay), on specific immune response (antibody production in response to vaccination), on the relative proportion of different types of white blood cells, on the structure of fish organs, on stress response and on osmoregulation.

The exposure to copper affected survival of rainbow trout. Mortalities were associated with concentrations of 20 μgL^{-1} of ASV-labile copper (a measure of biologically available copper) or 60 μgL^{-1} of total copper and occurred after more than two weeks of exposure. The growth of the fish was affected by six weeks exposure to about 2.7 μgL^{-1} of ASV-labile copper in one tank, but this concentration of copper did not affect the growth of fish in a second tank. Exposure to 8 μgL^{-1} or more of ASV-labile copper consistently affected growth. Although there was no statistically significant effect, the results suggested that antibody production against bacterial pathogens would be affected at all tested copper concentrations (exposure for seven weeks to average concentration of 3.29 μgL^{-1} or greater of ASV-labile copper). There did not seem to be any consistent relationship between exposure to copper and non-specific immune response of rainbow trout, measured by phagocytic activity of white blood cells. A statistically significant reduction in the percentage of circulating lymphocytes (white blood cells responsible for specific immune response) was observed in the fish exposed to copper for seven weeks. Elevated levels of sodium and potassium in the blood of fish exposed to higher concentrations of copper for seven weeks suggested osmoregulatory problems. The fish exposed to copper seemed to have altered structure of their gills, in particular an increased number of mucous cells, an increased number of chloride cells and an

increased thickness of respiratory epithelium were observed. There was a great difference between individual fish for most of the measurements.

Although this study investigated the effects of copper on rainbow trout, the results cannot be easily extrapolated to the situation in Macquarie Harbour because of differences in water chemistry. In both experiments the fish were exposed to copper only in the form of copper sulphate. The hydrochemistry of Macquarie Harbour is much more complex and although copper is the major obvious contaminant, fish may be also exposed to pH changes, other metals and changes in salinity. On the other hand, Macquarie Harbour water would most likely contain more organic matter and possibly humic acid or other compounds capable of binding copper and making it less available to the fish.

The adverse effects of copper on rainbow trout observed in this study and published literature indicate that caution should be applied if it is proposed to culture salmonid fish in those areas of Macquarie Harbour most affected by copper pollution. Although some of our results are not statistically significant and sometimes there is a lack of a dose response relationship, they should be treated as potential effects on rainbow trout exposed to copper in brackish water. Rainbow trout tested in this study seemed to be more susceptible to copper than reported in the literature, possibly as a result of salinity changes, which are not uncommon in Macquarie Harbour. Not only could the survival of the fish be affected by copper, but sub-lethal concentrations of copper may also reduce growth and lower resistance to infectious diseases or to salinity fluctuations.

The experiments tested the effects of copper only on rainbow trout. It should be noted that no experiments were done with Atlantic salmon, which is the second species cultured in Macquarie Harbour. Future research should include additional copper toxicity studies on rainbow trout and Atlantic salmon investigating potential effects of Macquarie Harbour water on the fish. The study described in this report could not address environmental factors other than copper level and salinity changes. Running the experiments in situ or using Macquarie Harbour water would be an advantage. Furthermore, research is necessary to determine the best analytical copper speciation method. As the effects detected in our experiments did not always relate to ASV-labile copper or total copper level in water, other measurements of copper speciation, such as free copper ion content in water should be used. Additionally, copper levels in Macquarie Harbour should be monitored on a regular basis, particularly in the vicinity of fish farms. Potential use of data loggers for water quality measurements, including copper concentrations, should be investigated.

Acknowledgments

The authors thank Warren Jones, Simon Stanley, Patrick McBride and Jon Bryan for their constructive comments on drafts of the manuscript, Barry Munday and Warren Jones for helpful discussions and Marianne Helders for help with ELISA. We would like to thank Annabel Tyson, Annette Daun and Rose Tremayne for their administrative assistance, and Robert Gurney, David Ransome and Belinda Williams for their technical assistance.

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1 Introduction

For a century, copper has been mined and processed at the Mount Lyell Mine at Queenstown in western Tasmania. Until the mine was taken over in 1994 by a new operator, Copper Mines of Tasmania Pty Ltd (CMT), scant regard had been paid to protecting the environment. During the previous 100 years of mining operations tailings, slag and acid drainage have been discharged into the rivers. As a consequence all aquatic life in the Queen and lower King Rivers has been killed and the banks smothered with tailings. Furthermore, the contamination affected Macquarie Harbour in which a delta of tailings the size of a city suburb has been created. The rivers and harbour have become contaminated with toxic metals, particularly copper, posing a potential hazard to the fishing industry and other harbour uses. The mining also affected terrestrial environment, for example vegetation on the hills surrounding Queenstown was destroyed through tree felling, fire erosion and toxic fumes from smelting. Smelting ceased in the 1960s, and CMT has built a dam to contain tailings. Acid drainage, largely resulting from past mining activities, remains a major environmental problem (McQuade et al 1995).

Macquarie Harbour is an almost entirely land-locked estuarine harbour. Most sites are well protected and the brackish water would reduce incidence of AGD (amoebic gill disease) which is the main health problem in sea culture of salmonids in Tasmania. Due to its geomorphology and hydrology Macquarie Harbour is an excellent site for cage farming of salmonid fish. Interest in marine farming in Macquarie Harbour was first expressed in 1987, only 18 months after the *Salt-Water Salmonid Culture Act* 1985. By 1993 a total of ten leases had been granted. The two species cultured in Macquarie Harbour are Atlantic salmon, *Salmo salar*, and rainbow trout, *Oncorhynchus mykiss*. A number of significant fish kills has been reported from some of the farms in northern part of Macquarie Harbour in the late 80s (Locher & Koehnken 1993) and anecdotal evidence implicated mine tailings, however no conclusive evidence was available. There is growing concern about the potential effects of pollution from the King River on the future of fish farming in Macquarie Harbour.

Although copper is an essential element, it is one of the most toxic heavy metals to fish. Speciation of copper as well as environmental conditions such as salinity, water hardness, alkalinity and presence of organic matter can greatly affect toxicity of copper to fish (Sorensen 1991). Additionally fish consumers may worry about potential copper accumulation in fish tissue. Although there is a lot of published information on copper effects on fish (for review see Sorensen 1991), most of the research has been done on early life stages of fish in a freshwater environment. Little information is available on the toxicity and accumulation of copper in farmed fish in brackish water.

The Mount Lyell Remediation Research and Demonstration Program has undertaken a series of comprehensive research projects to assess the environmental impact of metal release from past and present mining operations around Queenstown, as a part of development of a remediation strategy. This project was a part of the assessment of the biological impact of elevated copper levels on Macquarie Harbour. The main aim of this project was to investigate the effects of sub-lethal concentrations of copper on rainbow trout growth and health. The growth of the fish was estimated on the basis of wet weight gain. Additionally, protein content in white muscle and the RNA/protein ratio in white muscle were measured to detect changes which could affect fish growth in the long term (Busacker et al 1990). The health of the fish cannot be determined on the basis of measuring a single variable (Anderson 1990). Measurements of immune response (both nonspecific and specific), structural changes in organs and tissues as well as stress response should be included to estimate fish health. Acute

stress response was estimated using the cortisol level in blood, macrophage assay assessed nonspecific immune response, differential white blood cell count was used to determine both specific and nonspecific, antibody production determined specific immune response, sodium level in blood plasma assessed osmoregulatory stress and histology established any structural changes in organs of the fish.

2 Materials and methods

2.1 Fish and experimental procedures

2.1.1 Experiment 1

Rainbow trout (*Oncorhynchus mykiss*) of length 16.1–22.3 cm and weight 65.24–133.24 g were obtained from Sevrup Fisheries at Cressy and held in freshwater until time of exposure.

For the exposure period of six weeks, groups of 15 fish were tagged and placed in eight 250 L tanks in a flow through system. Salinity was kept at 19.6 ppt (standard deviation 1.1) and the flow rate through each tank was 0.7 L min^{-1}. Seawater was collected by Hodges Transport and delivered twice weekly. Freshwater used to mix to obtain the correct salinity was from the Launceston water supply and was first tested for copper content which was found to be negligible. Throughout the exposure period temperature ranged from 14.0–16.7°C, mean 15.29°C (standard deviation 0.76°C), pH 7.19–8.33, mean 7.62 (standard deviation 0.21), ammonia < 0.1mgL^{-1} and dissolved oxygen 9.5–12.5 mgL^{-1}, mean 11.53 (standard deviation 0.19 mgL^{-1}). Fish were fed with Gibsons Trout Feed at a rate of 1% body weight per day.

Analy-tical grade copper sulphate ($CuSO_4.5H_2O$) was used to prepare test concentrations. Test fish were exposed to three nominal copper concentrations (30 μgL^{-1}, 70 μgL^{-1} and 200 μgL^{-1}). Stock solutions were made up to 3 mg, 7 mg and 20 mgL^{-1} and delivered to their respective tanks by gravity flow at a rate of 7 mL min^{-1}. Two replicate tanks were used for each concentration and two control tanks without metal were kept under similar conditions. Any mortalities were replaced with new fish to maintain the same biomass for growth study. However, the fish which were exposed to copper for less than 6 weeks were not taken into account for the evaluation of effects of six week exposure to copper on rainbow trout.

At the end of the experiment the fish were anaesthetised using 2-phenoxyethanol, blood was collected for cortisol analysis, then the fish were weighed and revived. After about 1 hour the fish were anaesthetised and injected with yeast for the estimation of *in vivo* phagocytosis. Two hours later the fish were sacrificed using an overdose of 2-phenoxyethanol. The fish were dissected and tissue was collected for analysis (table 1).

2.1.2 Experiment 2

Rainbow trout (*Oncorhynchus mykiss*) of weight 110–375.1 g were obtained from Sevrup Fisheries at Cressy and held in freshwater until the time of exposure.

For the exposure period of seven weeks, groups of 10 fish were tagged and placed in eight 250 L tanks in a flow through system. Salinity was kept at 14.6 ppt (standard deviation 6.8, range 0–23) and the flow rate through each tank was 0.7 L min^{-1}. Seawater was collected by Hodges Transport and delivered twice weekly. Due to interruptions in the delivery which were out of the control of the University of Tasmania the water salinity fluctuated more than planned and on one occasion it reached 0 ppt. Freshwater used to mix to obtain the correct salinity was from the Launceston water supply and was first tested for copper content which was found to be negligible. Throughout the exposure period temperature ranged from 9.2–18.3°C, mean 13.4°C

(standard deviation 1.7°C), pH 6.5–9.3, mean 7.2 (standard deviation 0.39), ammonia < 0.1mgL^{-1} and dissolved oxygen 5.2–13.6 mgL^{-1}, mean 7.95 (standard deviation 2.19 mgL^{-1}). Fish were fed with Gibsons Trout Feed at a rate of 1% body weight per day.

Table 1 Endpoints used in experiments 1 and 2 to determine the effects of copper on rainbow trout

Endpoint	Experiment 1	Experiment 2
growth (weight gain)	Y	N
protein level	Y	N
nucleic acid	Y	N
cortisol	Y	N
sodium level in blood	N	Y
macrophage assay	Y	Y
WBC differential count	Y	Y
antibody production	N	Y
copper in liver	Y	N
histology	Y	N

Analytical grade copper sulphate ($CuSO_4.5H_2O$) was used to prepare test concentrations. Test fish were exposed to three nominal copper concentrations (30 µgL^{-1}, 70 µgL^{-1} and 200 µgL^{-1}). Stock solutions were made up to 3 mg, 7 mg and 20 mgL^{-1} and delivered to their respective tanks by gravity flow at a rate of 7 mL min^{-1}. Two replicate tanks were used for each concentration and two control tanks without metal were kept under similar conditions.

After 3 weeks of exposure the fish were anaesthetised and injected intraperitoneally with 0.1 mL of *Vibrio* vaccine. Formalin killed whole cells of *Vibrio anguillarum* were obtained from Department of Primary Industry and Fisheries, Mt Pleasant Fish Health Unit. A suspension of 10^6 organisms/0.1 ml phosphate buffered saline (PBS) was used and injected into fish via intraperitoneal injection after 3 weeks exposure to copper. Four weeks later fish were anaesthetised using 2-phenoxyethanol (0.3 ml L^{-1}) and blood samples were collected from the caudal vein. Blood was centrifuged and the plasma collected and stored at -20°C. The fish were anaesthetised one hour later and injected with yeast for the estimation of *in vivo* phagocytosis. Two hours later the fish were sacrificed using an overdose of 2-phenoxyethanol. The fish were dissected and tissue was collected for analysis (table 1).

2.2 Measured concentrations of copper in water

The levels of copper in the water were measured as total and anodic stripping voltammetry (ASV)-labile copper. Up to ten water samples were collected from each exposure tank during the experiments and analysed for copper content. Total copper in the unfiltered samples and total dissolved copper in the filtered samples were determined by graphite furnace atomic absorption spectroscopy (GFAAS) at Department of Environment and Land Management, Hobart. ASV-labile copper was measured using anodic stripping voltammetry.

2.3 Fish growth

Fish growth was assessed on the basis of wet weight gain, protein content in white muscle, RNA concentration in white muscle and RNA to protein ratio. The wet weight gain was calculated as the difference between the weight of the fish at the end of 6 weeks exposure and

the weight of the fish at the beginning of the exposure, at the time of stocking the tanks. The fish were individually tagged to allow for this procedure. Protein concentration (mg protein/g of tissue) was measured using a modified Folin phenol method (Lowry et al 1951, Schacterle & Pollock 1973). Nucleic acids were extracted by a modification of the Schmidt-Thannhauser method (Munro & Fleck 1966). RNA concentration (mg RNA/g of tissue) and DNA concentration (mg DNA/g of tissue) were estimated using dual absorbance measurements at 260 nm and 232 nm (Ashford & Pain 1986).

2.4 Cortisol level in blood plasma

Plasma levels of cortisol were measured by radioimmunoassay (RIA). Fifty mL of plasma were extracted with 1 mL of ethyl acetate. Extraction efficiency was calculated as recovery of 3H-labeled cortisol extracted with 50 mL of plasma. The extraction efficiency was 97.7% (n=3) and the assay values were corrected accordingly.

For hormone measurements, 50 mL of extract was evaporated overnight and redissolved in 200 mL of 0.05 M phosphate buffer containing 0.1% gelatine and incubated overnight with 200 mL of 3H-labeled cortisol and 200 mL of antibody. After incubation samples were cooled on ice for 10 minutes, then 200 mL of cold dextran coated charcoal was added, and the tubes were vortexed and left to stand for 10 minutes after the last addition of charcoal before centrifuging at 3000 rpm at 4°C for 10 minutes. After decanting the supernatant was combined with 4 mL of scintillation cocktail for counting (Pankhurst & Carragher 1992).

Duplicate samples were assayed and interassay variability was measured using a pooled steroid giving a %CV of 10.3%.

2.5 Sodium level in blood plasma

The level of sodium in blood was determined using atomic absorption spectrometry. Blood plasma (100 mL) was placed in a 5 mL standard volumetric flask. Ionisation suppressant (0.5 mL of a 10 000 mgL^{-1} solution of strontium) was added and the mixture was made up to volume with ultra pure water. Standards were prepared in the range of 10–200 mgL^{-1} of sodium and potassium. The determination was carried out on a Varian SPECTRAA300 Atomic Absorption Spectrometer used in the mode of Flame Emission.

2.6 Phagocytosis *in vivo*

The method was based on phagocytosis *in vivo* assay described by Peters et al (1991). Yeast solution (*Saccharomyces cerevisiae*) was prepared to a concentration of 10^8 yeast/ml in PBS. Five ml of Congo red was added to 5 ml of yeast solution and autoclaved. Yeast cells were washed in PBS and resuspended in the same volume of PBS before use.

Fish were anaesthetised using 2-phenoxyethanol (0.3 ml/L) and injected via the caudal vein with 0.1 ml/100 g body weight yeast solution. They were revived and kept for two hours. Fish were then killed with a lethal dose of 2-phenoxyethanol and the head kidney removed. Each kidney was placed in a plastic petri dish containing 3–4 mL PBS and teased through stainless steel mesh (0.3 mm) to remove clumps. The solution was transferred to a test tube and allowed to settle for 10 minutes. Two ml of FICOLL was placed in 10 ml polypropylene centrifuge tubes. The macrophage solution was carefully added and centrifuged at 400 g for 20 minutes.

The macrophage (interface) layer was removed with pasteur pipette and washed in PBS. One hundred phagocytes containing yeast cells were counted and the average number of yeast cells / macrophage calculated for each fish.

2.7 Differential WBC counts

Slides with blood smears were stained with DiffQuick and examined under a microscope. One hundred white blood cells were identified using the battlement scanning method. The percentage of lymphocytes, neutrophils, monocytes and thrombocytes was determined.

2.8 Circulating antibody level

Plasma antibody levels were measured by enzyme-linked-immunosorbent-assay (ELISA). *Vibrio* antigen was diluted to 10 mg/ml in coating buffer (TS.methanol) and put in each well of two Nunc-Immunoplates using transferpette-12 and incubated overnight at 4°C. Excess antigen was removed and blocking solution (PBS/3%casein) was added for 30 minutes at room temperature. Plates were washed 3 times in a Biorad-immunowash using PBS/0.05% tween-20.

Test sera was diluted 1:100 in PBS/1%tween-20 and added. Blank, positive and negative samples were included on each plate and each sample was replicated and left for 90 minutes at room temperature. After washing 3 times the plates were incubated with Mouse-anti-Rainbow trout monoclonal antibody, diluted 1:10 in PBS/1% tween-20 for 90 minutes at room temperature. Plates were washed 3 times. Conjugate Rabbit-anti-Mouse-HPR diluted 1:1000 in PBS/1% tween-20 was added for 90 minutes at room temperature. Plates were washed 5 times. Colour developing solution OPD (1 tablet / 37.5 ml Sodium Citrate-Phosphate buffer + 0.012%H_2O_2) was added for 10 minutes. The reaction was then stopped with 3N HCl.

Optical density (OD) was measured using a Titertekplus microplate reader at 492 nm wavelength.

2.9 Histology

Small pieces of organs were dissected from fish and immediately fixed in 10% phosphate buffered formalin. The tissue was then routinely processed for histology, with sections cut at 5 μm and stained with haematoxylin-eosin. The slides were examined using a Leica microscope using magnification of 600. Ten fish were examined from each tank and the results were quantified as prevalence (percentage of fish affected).

2.10 Copper content in liver

Individual livers were freeze dried (DynaVac Mini Ultra Cold) to constant mass. Approximately 50 mg of freeze dried liver was weighed accurately into acid washed 25 mL Erlenmeyer flasks. Individual livers were analysed separately.

One mL of AR HNO_3 was added to the flask and a watch glass was placed over the mouth of the flask. The sample was allowed to digest overnight by which time near total dissolution of the sample was achieved. The samples were then heated to reflux for 1.5 to 2.0 hours on a hotplate until the evolution of oxides of nitrogen ceased. One mL of AR H_2O_2 was added to the hot digest in 0.2 mL aliquots, and the solution was then cooled to room temperature. One mL of 10% HNO_3 was added and the flask warmed gently for 15 minutes. The flask was 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 AA (Varian Spectra AA 300) which was calibrated with a matrix matched standard and a 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) were carried through with each batch of samples.

2.11 Statistical analyses

The results were analysed using analysis of variance (ANOVA). Results were considered statistically significant if $P<0.05$. Before ANOVA was performed Cochran's test was used to determine if the variances were homogenous. In the case of unbalanced design Bartlett's test was used instead of Cochran's. If the variances were heterogenous the data were transformed and resubmitted to the test. The raw data were analysed and the results interpreted with caution if despite transformations the variances were heterogenous. When 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). Relationships between two variables were assessed using correlation analysis.

3 Results

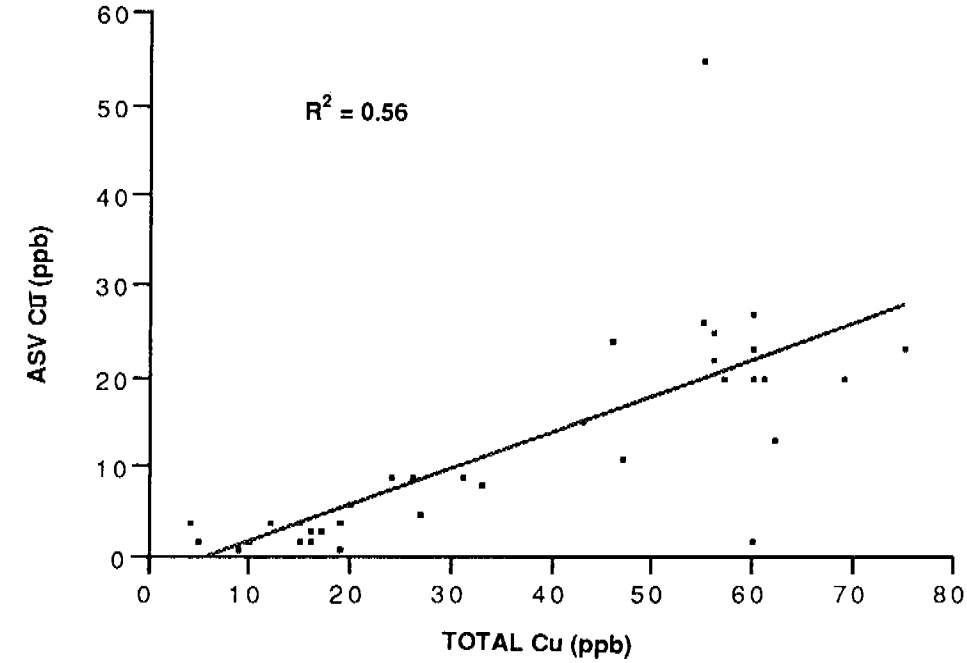
3.1 Measured concentrations of copper in water

The measured concentrations of total copper were much lower than nominal concentrations. However, concentrations of stock solutions were very close to nominal and the delivery rate of the stock solutions as well as water flow was closely monitored and on most occasions was the same as planned. Copper distribution in exposure tanks was very uniform and no sites of higher copper concentrations could be detected within the tanks. Thus the cause of the discrepancy between the nominal and measured copper concentrations was not established. As there were unexpected fish mortalities (see 3.2) even at these lower copper concentrations no attempt was made to increase the measured concentrations.

In the first experiment the levels in control tanks were below detection limit, the mean levels in the treated tanks ranged from 10.75 μgL^{-1} to 46.77 μgL^{-1} of total copper and 2.71 μgL^{-1} to 21.00 μgL^{-1} of ASV-labile copper (table 2). The means were based on eight to ten measurements for total copper and six to nine measurements for ASV-labile copper from each tank. There was high variation between measurements. The greatest measured total copper concentration was 69 μgL^{-1}.

In the second experiment the levels of copper in control tanks were below detection limit, while the mean levels in the treated tanks ranged from 16.00 μgL^{-1} to 70.75 μgL^{-1} of total copper and 3.29 μgL^{-1} to 68.00 μgL^{-1} of ASV-labile copper (table 2). These means were based on four measurements for each tank. There was high variation within each tank.

The results for ASV-labile copper seemed to be related to the levels of total copper in the same sample. There was a statistically significant positive correlation between total and ASV-labile copper levels in both experiments ($P = 0$, fig 1). The results from experiment 1 for total and ASV-labile copper were significantly correlated ($R^2 = 0.5589$, $y = 0.3979 x - 2.1513$) as were the results from experiment 2 ($R^2 = 0.9614$, $y = 0.4861 x - 3.609$). In some cases, particularly in the second experiment, the levels of ASV-labile copper were very close to the total copper level in the water, which means that most of the copper was biologically available. However, in most cases the concentrations of ASV-labile copper were less than 25% of total copper. There was high variability for ASV-labile copper within each tank.



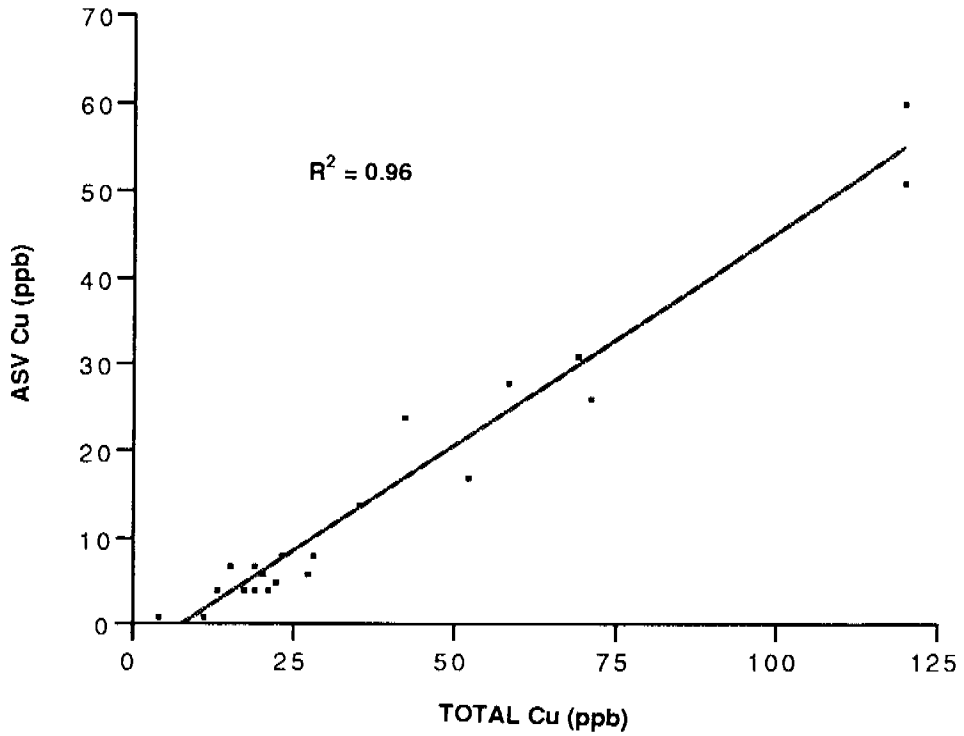


Figure 1 Relationship between total copper level and ASV-labile copper level in water samples from experiments 1 and 2

Measured concentrations of copper are used in the following results (section 3) and discussion (section 4). As ASV-labile copper better represents biologically available copper the effects are related to the average ASV-labile copper levels recorded. Due to the differences in exposure levels between tanks (table 2), the tanks were not used as replicates and the differences between tanks were related to the differences in copper concentrations in each tank.

Table 2 Nominal and measured concentrations of total and ASV-labile copper in exposure tanks in experiments 1 and 2. The data are presented as a mean and (standard error).

nominal Cu (μgL^{-1})	exp 1 total Cu (μgL^{-1})	exp 1 ASV Cu (μgL^{-1})	exp 2 total Cu μgL^{-1})	exp 2 ASV Cu μgL^{-1})
30	22.00 (5.98)	8.20 (5.66)	17.50 (4.84)	3.29 (0.71)
30	10.75 (2.83)	2.71 (0.47)	16.00 (3.11)	3.50 (1.07)
70	17.12 (2.83)	4.43 (1.30)	33.25 (13.32)	9.63 (3.81)
70	12.6 (2.26)	2.78 (0.95)	43.50 (16.56)	8.25 (3.57)
200	41.55 (9.13)	21.00 (2.20)	44.50 (12.1)	45.25 (22.53)
200	46.77 (7.98)	19.5 (2.19)	70.75 (28.56)	68.00 (30.98)

3.2 Fish survival

In the first experiment, except for the fish exposed to the highest concentrations of copper the survival was 100% (table 4). In tank 4 (mean concentration of 21 μgL^{-1} of ASV-labile copper) the survival was 80% and in tank 8 (mean concentration of 19.5 μgL^{-1} of ASV-labile copper) the survival was 73%. In both tanks all the mortalities happened 3 weeks after the exposure started and the fish died within 24 hours from each other. The copper level at the time of mortality was: in tank 4 total copper 56 μgL^{-1} and ASV-labile copper 22 μgL^{-1} and in tank 8 total copper 60 μgL^{-1} and ASV-labile copper 20 μgL^{-1}.

In the second experiment survival was much lower, particularly in the higher concentrations of copper (table 4). The concentration recorded on one of the days when mortalities occurred was 58 μgL^{-1} of total copper and 28 μgL^{-1} of ASV-labile copper.

In both experiments the mortality of fish seemed to be associated with ASV-labile copper level being greater than 20 μgL^{-1}. The levels of total copper at the time of mortalities were at or above of 60 μgL^{-1}.

Table 3 Percentage survival in the treated tanks in experiments 1 and 2. Results are presented as mean and (standard error).

exp 1 ASV Cu (μgL^{-1})	exp 1 survival (%)	exp 2 ASV Cu (μgL^{-1})	exp 2 survival (%)
8.20 (5.66)	100	3.29 (0.71)	100
2.71 (0.47)	100	3.50 (1.07)	80
4.43 (1.30)	100	9.63 (3.81)	70
2.78 (0.95)	100	8.25 (3.57)	40
21.00 (2.20)	80	45.25 (22.53)	20
19.5 (2.19)	73	68.00 (30.98)	40

3.3 Fish growth

The growth as measured by wet weight gain seemed to be reduced in fish from some of the tanks exposed to copper (table 4). The variation between fish and tanks was high (fig 2). The fish from different tanks showed significantly different wet weight gain (ANOVA, P=0).

The relationship between wet weight gain, white muscle protein concentration, RNA concentration and RNA:protein ratio ('the capacity for protein synthesis') was also examined. It was hypothesised that at higher copper concentrations protein turnover would be greater to repair damaged proteins. One mechanism which would facilitate this increase would be through a higher RNA concentrations as indicated by direct measurements or by greater RNA:protein ratio.

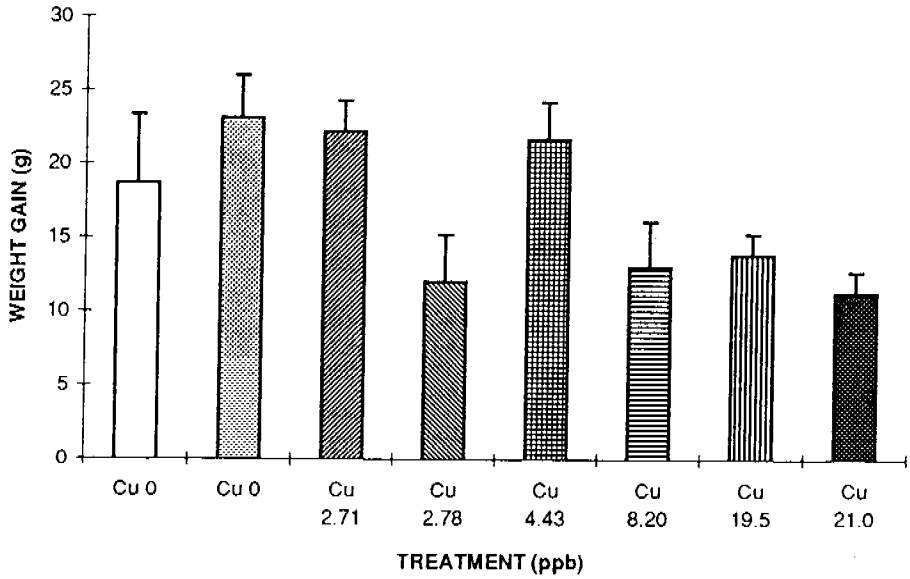
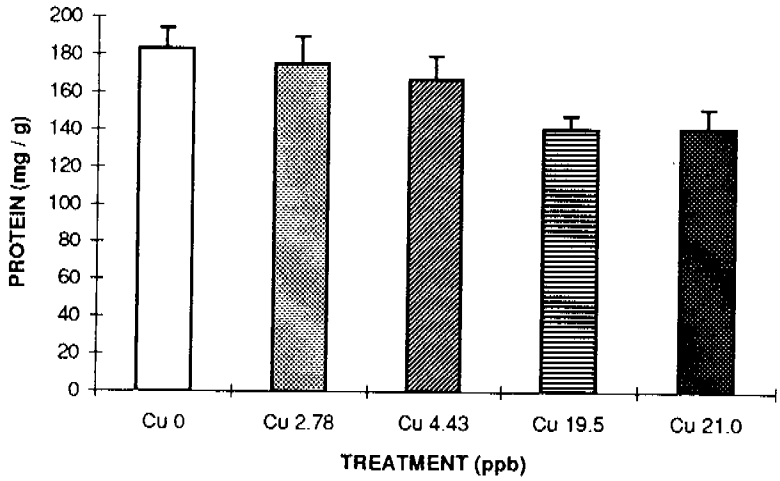
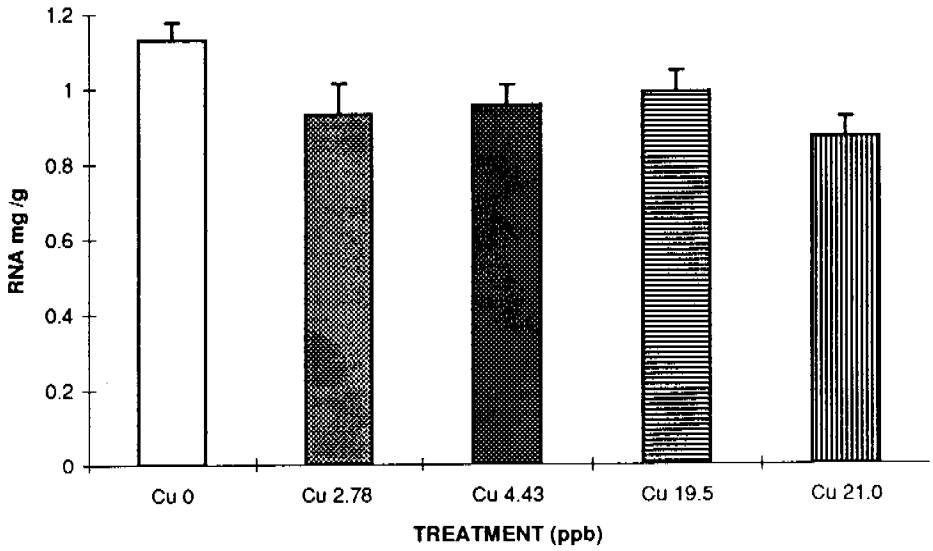


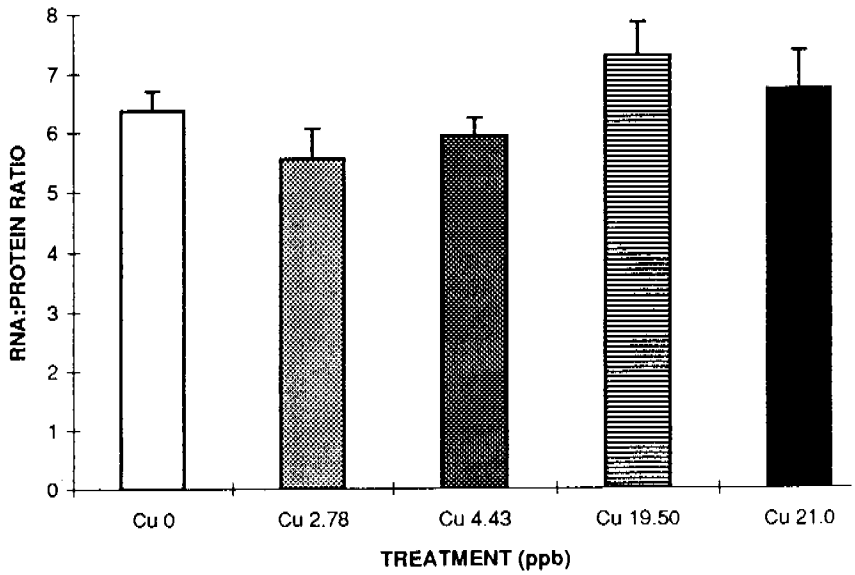
Figure 2 Effect of six weeks exposure to copper on growth of rainbow trout
a: effect on wet weight gain



b: effect on protein content in white muscle



c: effect on RNA content in white muscle



d: effect on RNA:protein ratio in white muscle

Weight gain decreased as copper concentration increased and this trend was reflected by white muscle protein concentration, which also decreased (table 4, fig 2). The protein level in the muscle of fish from tanks with copper concentration greater than 20 μgL^{-1} was significantly lower than in the control group or fish exposed to lower concentrations of copper (ANOVA, P=0.0182). White muscle RNA concentration did not show a clear pattern although it was greater in the control fish (control fish 1.13 mg/g, treated 0.87–0.99 mg/g, fig 2). There was a statistically significant effect of exposure to copper on RNA content in white muscle (ANOVA, P=0.0368). The fish exposed to copper, except for the fish in tank with an average ASV-labile copper level of 19.5 μgL^{-1} showed significantly lower RNA level in white muscle than the control fish.

Compared with the control (6.4) the RNA:protein ratio was lower at the lower copper concentrations (5.5 and 5.9 for fish exposed to 2.78 μgL^{-1} and 4.43 μgL^{-1} of ASV-labile copper) but greater in the fish exposed to the greater copper concentrations (6.7 and 7.3 for fish exposed to around 20 μgL^{-1} of ASV-labile copper, fig 2). However, there was no evidence for statistically significant effect of exposure to copper on RNA:protein ratio in white muscle (ANOVA, P=0.1360).

Table 4 Effects of copper on growth of fish measured as wet weight gain, protein content in white muscle and RNA:protein ratio in white muscle (experiment 1). Results shown as mean and (standard error); NA: results not available.

exp 1 ASV Cu (μgL^{-1})	wet weight gain (g)	protein content (mg g^{-1})	RNA:protein
0 (0)	23.19 (2.88)	183.14 (10.63)	6.37 (0.33)
0 (0)	18.74 (4.63)	NA	NA
2.71 (0.47)	22.28 (2.07)	NA	NA
2.78 (0.95)	12.03 (3.19)	175.15 (14.37)	5.54 (0.52)
4.43 (1.30)	21.73 (2.54)	167.20 (12.02)	5.93 (0.31)
8.20 (5.66)	13.04 (3.10)	NA	NA
19.50 (2.19)	13.88 (1.43)	140.53 (7.26)	7.29 (0.56)
21.00 (2.20)	11.32 (1.36)	141.00 (10.38)	6.72 (0.66)

3.4 Cortisol level in blood plasma

Cortisol level seemed to be elevated in blood plasma of many individual fish, possibly due to social hierarchy established in each tank (table 5, fig 3). Only fish from one tank (19.5 μgL^{-1} of ASV-labile copper) had low levels of cortisol (<10 ng/mL). The variation between fish and tanks was high (fig 3). The cortisol level of fish from different tanks was significantly different (ANOVA, P=0.008).

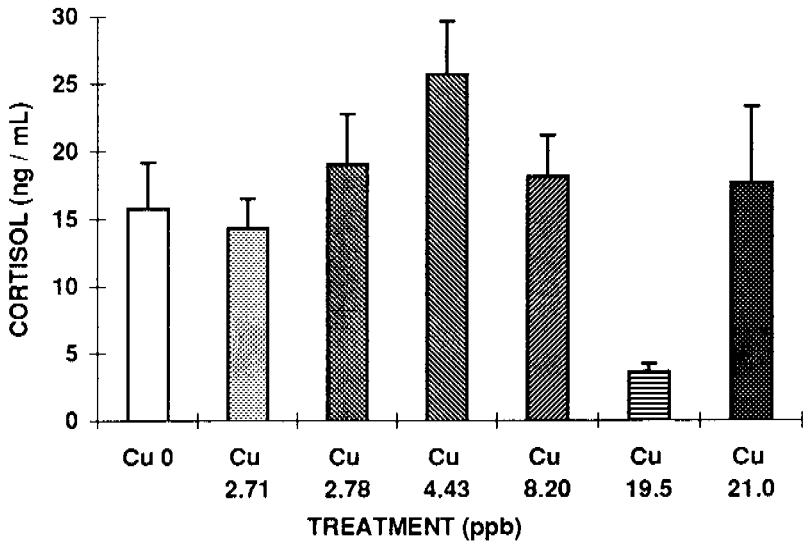


Figure 3 Effect of six weeks exposure to copper on cortisol level in blood plasma of rainbow trout

Table 5 Cortisol level in blood plasma of rainbow trout (experiment 1). Results are presented as mean and (standard error).

exp 1 ASV Cu (μgL^{-1})	number of fish tested	cortisol (ng/mL)
0 (0)	1	<2.46
0 (0)	15	15.80 (3.40)
2.71 (0.47)	11	14.35 (2.19)
2.78 (0.95)	11	19.08 (3.70)
4.43 (1.30)	13	25.67 (4.01)
8.20 (5.66)	10	18.12 (3.07)
19.50 (2.19)	12	3.62 (0.63)
21.00 (2.20)	14	17.63 (5.63)

3.5 *In vivo* phagocytosis

In both experiments there appeared to be no effect of exposure to copper on phagocytic activity of macrophages (table 6). There was a statistically significant difference between different tanks in the first experiment (ANOVA, P=0). The variation between fish and tanks was high (fig 4). Fish from one of the control tanks and fish from the tanks exposed to 2.7 μgL^{-1}, 8.2 μgL^{-1} and 19.5 μgL^{-1} of ASV-labile copper showed suppressed phagocytic activity of their head kidney macrophages whereas the phagocytic activity of macrophages from fish from the other tanks seemed to be enhanced (table 6, fig 4). The response to exposure to copper seemed to be inconsistent.

In the second experiment there was also a statistically significant difference between tanks (ANOVA, P=0.003). The fish exposed to 3.29 μgL^{-1} and 8.25 μgL^{-1} of ASV-labile copper showed reduced phagocytic activity of their macrophages and the fish exposed to 3.5 μgL^{-1} and 68 μgL^{-1} of ASV-labile copper showed increased phagocytic activity of their macrophages (fig 4). Again, the effects of exposure to copper were very inconsistent.

3.6 Differential white blood cell counts

In the first experiment lymphocytes comprised the majority of white blood cells in all fish examined with neutrophils being only 0.9% to 2.9% of all white blood cells (fig 5). The statistical tests failed to detect any effect of exposure to copper on percentages of lymphocytes (ANOVA, P=0.97) or neutrophils (ANOVA, P=0.88). However, the percentage of monocytes without any yeast cells was significantly greater in the fish exposed to 20 μgL^{-1} of ASV-labile copper (ANOVA, P=0).

In the second experiment the composition of white blood cells was similar as in experiment 1 (fig 5), however there was a statistically significant effect of the exposure to copper on the percentage of lymphocytes (ANOVA, P=0.0003) and monocytes (ANOVA, P=0.0017) but not neutrophils (ANOVA, P=0.3607) or thrombocytes (ANOVA, P=0.1674). The percentage of lymphocytes was significantly lower in the blood of fish exposed to copper and the percentage of monocytes was significantly greater after exposure to copper (fig 5). There was no consistent effect of the level of copper to which the fish were exposed.

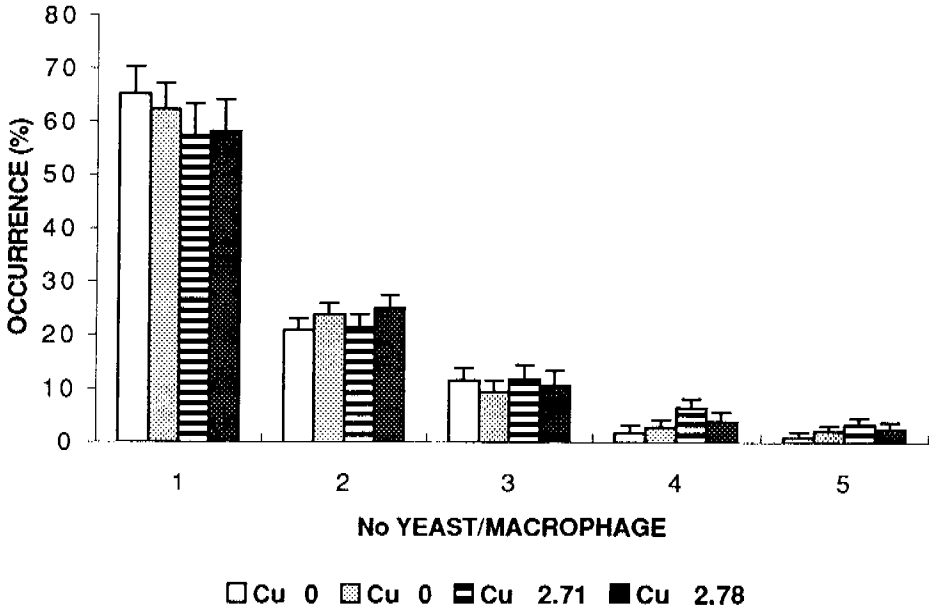
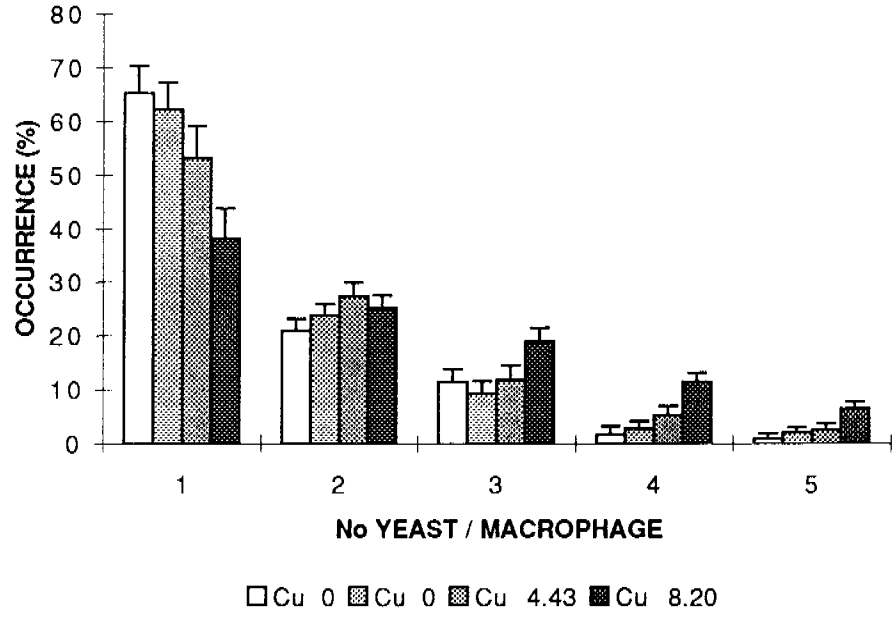
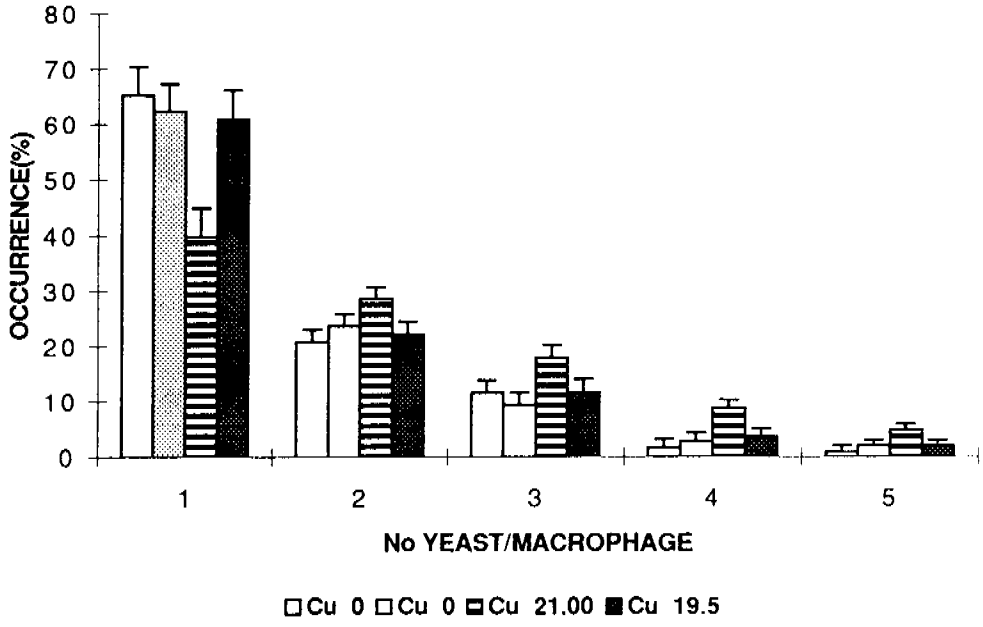


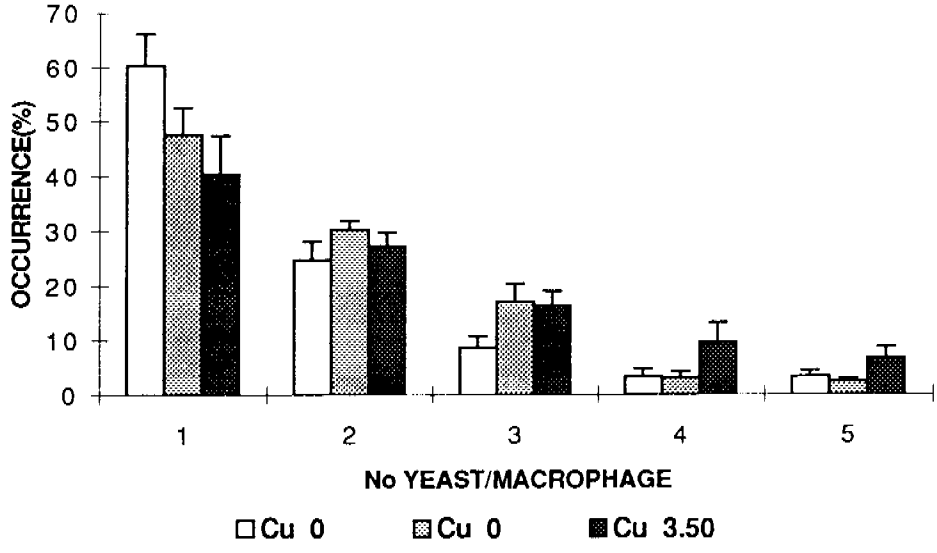
Figure 4 Effect of exposure to copper on *in vivo* phagocytosis by head kidney macrophages of rainbow trout
a: effect of six week exposure to low copper level on proportion of macrophages containing different numbers of yeast cells



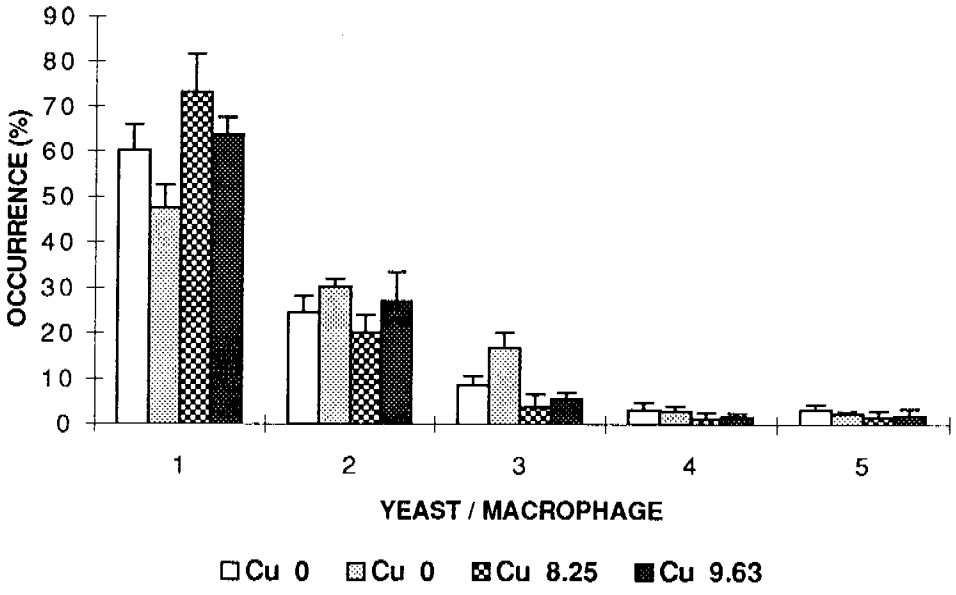
b: effect of six week exposure to medium copper level on proportion of macrophages containing different numbers of yeast cells



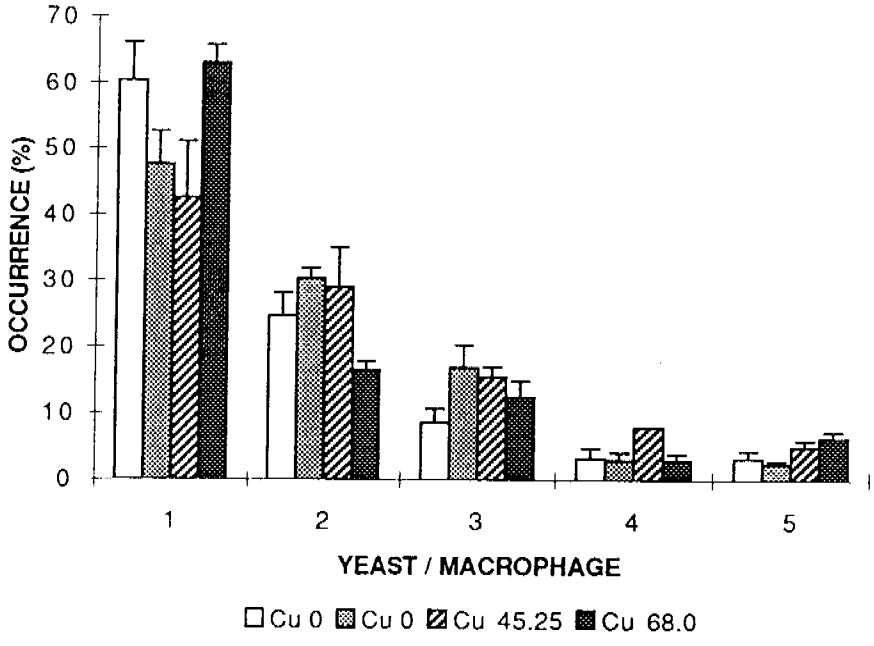
c: effect of six week exposure to high copper level on proportion of macrophages containing different numbers of yeast cells



d: effect of seven week exposure to low copper level on proportion of macrophages containing different numbers of yeast cells



e: effect of seven week exposure to medium copper level on proportion of macrophages containing different numbers of yeast cells



f: effect of seven week exposure to high copper level on proportion of macrophages containing different numbers of yeast cells

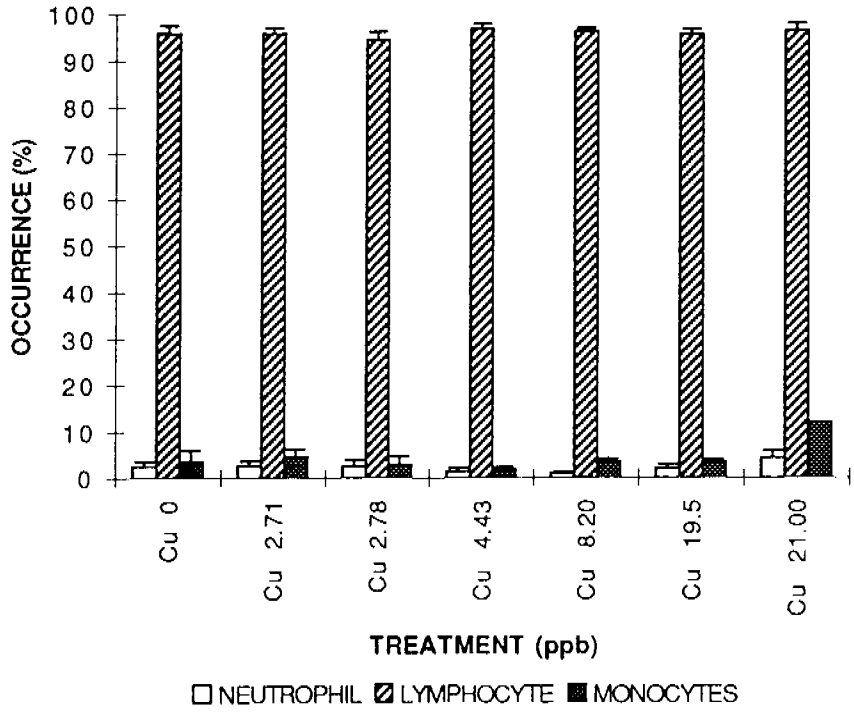
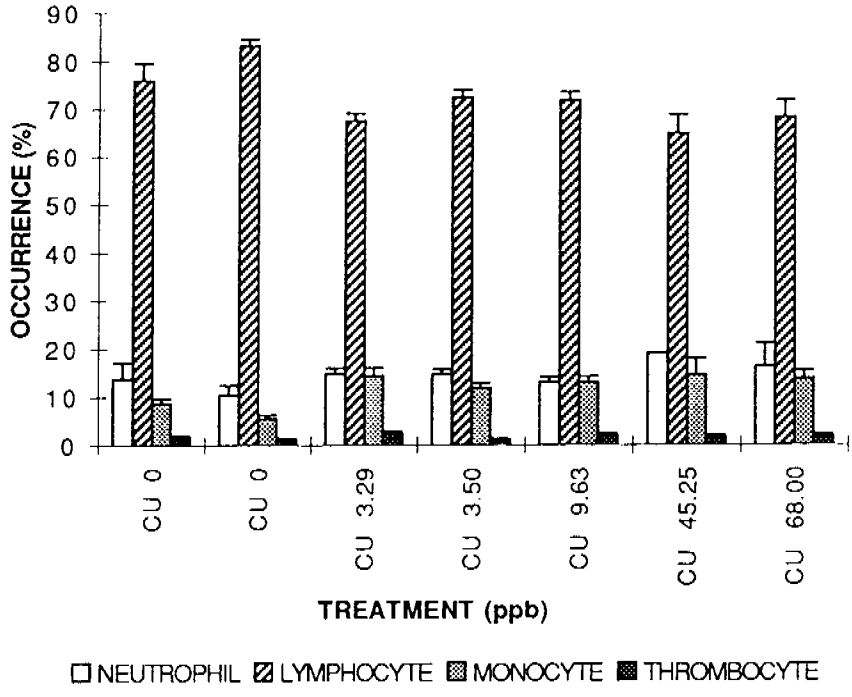


Figure 5 Effect of exposure to copper on differential white blood cell counts
a: experiment 1



b: experiment 2

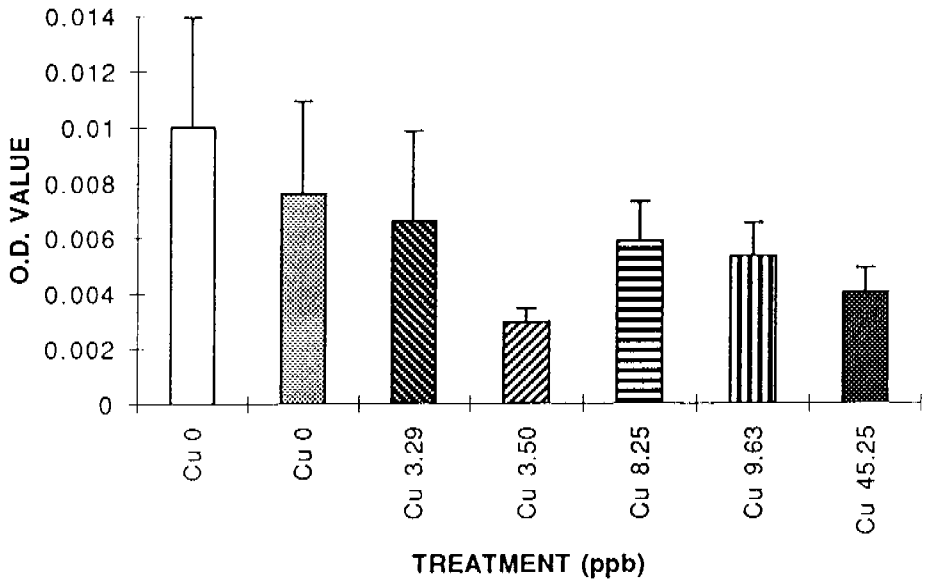


Figure 6 Effect of seven week exposure to copper on circulating antibody level in rainbow trout

Table 6 Effect of exposure to copper on *in vivo* phagocytosis by head kidney macrophages of rainbow trout (experiment 1 and 2). The results are presented as mean and (standard error).

exp 1 ASV Cu (μgL^{-1})	number of fish tested	number of yeast/ macrophage	exp 2 ASV Cu (μgL^{-1})	number of fish tested	number of yeast/ macrophage
0 (0)	13	2.02 (0.04)	0 (0)	7	1.92 (0.14)
0 (0)	13	1.54 (0.06)	0 (0)	8	1.71 (0.13)
2.71 (0.47)	9	1.65 (0.09)	3.29 (0.71)	2	1.28 (0.26)
2.78 (0.95)	10	2.24 (0.14)	3.50 (1.07)	7	2.26 (0.14)
4.43 (1.30)	13	2.30 (0.16)	9.63 (3.81)	6	1.37 (0.15)
8.20 (5.66)	9	1.78 (0.13)	8.25 (3.57)	3	1.42 (0.21)
19.50 (2.19)	10	2.08 (0.07)	45.25 (22.53)	2	2.02 (0.26)
21.00 (2.20)	9	1.73 (0.14)	68.00 (30.98)	4	1.73 (0.18)

3.7 Circulating antibody level

Although there seemed to be a trend of decreasing antibody level in fish exposed to copper, there was no statistically significant effect of the exposure to copper on antibody levels in fish (ANOVA, P=0.4833). There was very high individual variation, except for the fish exposed to highest copper concentration (fig 6).

3.8 Histology

No significant changes were observed in organs other than gills (table 7). Although there was a lot of individual variation, there appeared to be an effect from exposure to copper on gill structure.

Table 7 Prevalence of structural changes in gills of rainbow trout exposed to copper for six weeks

ASV-labile copper exposure (μgL^{-1})	increased number of mucous cells (%)	increased thickness of respiratory epithelium (%)	increased number of chloride cells (%)
0	20	20	30
0	30	10	20
2.7	80	70	40
2.8	50	0	10
4.4	60	20	20
8.2	60	0	30
19.5	80	10	40
21.0	70	30	40

Most fish exposed to copper seemed to have an increased number of mucous cells and sometimes chloride cells. Mucous cells were present in filamentar epithelium and also in the respiratory epithelium. In the gill of treated fish, chloride cells appeared to be necrotic. Some increase in respiratory diffusion distance due to thickening of respiratory epithelium was also observed. This was mostly due to swelling (hypertrophy) of individual cells, although proliferation of epithelial cells was also sometimes observed.

Livers of fish exposed to copper seemed to be more vacuolated than control fish, which seemed to have more 'feathery' appearance of hepatocytes. This may be due to greater accumulation of lipid within the livers of treated fish. Two fish exposed to copper had a depleted spleen.

3.9 Sodium and potassium level in blood

Although sodium levels seem to be elevated in the fish exposed to highest concentrations of copper (fig 7), there was no statistically significant effect (ANOVA, P=0.3410). The individual variation was high.

The exposure to copper had a statistically significant effect on the potassium level in fish blood (ANOVA, P=0). The fish exposed to the greatest copper concentrations (9.6 μgL^{-1} of ASV-labile copper and 68 µgL-1 of ASV-labile copper) had significantly elevated potassium levels relatively to controls and fish exposed to lower copper concentrations (fig 7).

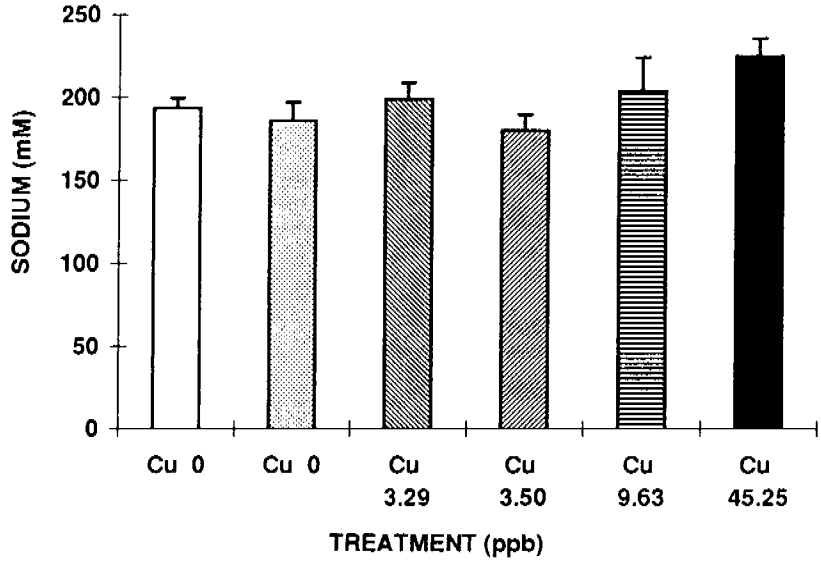
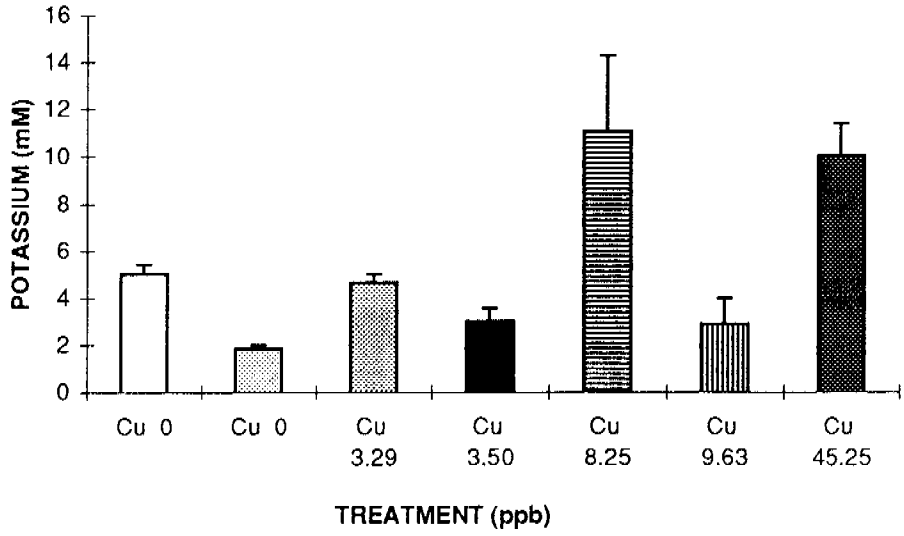


Figure 7 Effect of seven week exposure to copper on ion level in blood plasma
a: sodium



b: potassium

3.10 Copper residue in liver

There was a statistically significant difference between fish from different tanks (ANOVA, P=0.0132). The fish from one of the tanks exposed to 8.2 μgL^{-1} had a significantly greater copper content in their livers than control fish or fish exposed to copper in other tanks. There did not seem to be any relationship between exposure to copper and copper content of fish liver (fig 8). The copper content ranged from 0.04 to 0.86 mg/g dry weight. The average copper content was 0.41 and 0.44 mg/g dry weight for control tanks, 0.33 and 0.37 mg/g dry weight for 2.7 μgL^{-1} of ASV-labile copper exposure, 0.32 and 0.47 for 4.4 μgL^{-1} and 8.2 μgL^{-1} of ASV-labile copper exposure, 0.40 and 0.33 mg/g dry weight for 20 μgL^{-1} of ASV-labile copper exposure. The variation between fish and tanks was high (fig 8).

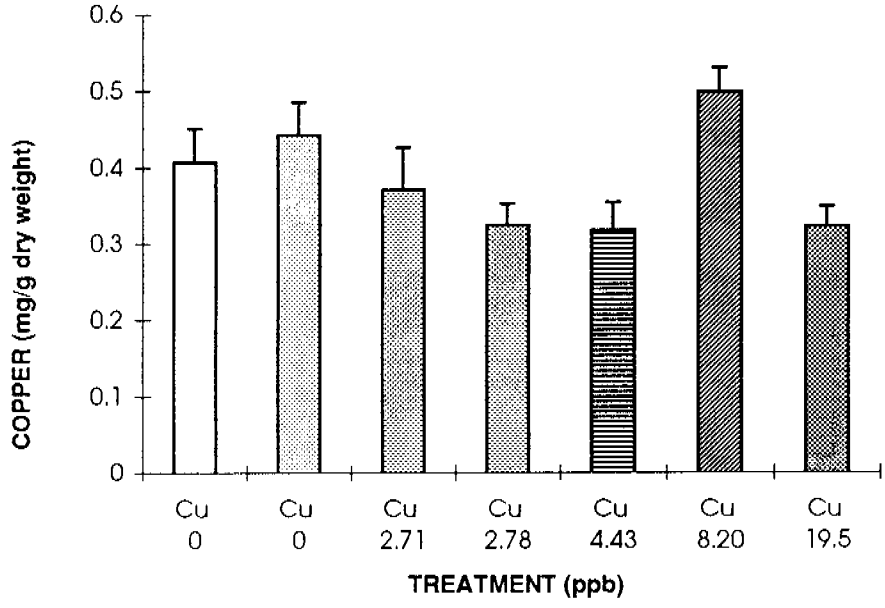


Figure 8 Effect of six week exposure to copper on copper residue level in fish liver

4 Discussion

4.1 Copper concentration in water

Copper speciation in water has a great effect on the toxic effects on aquatic organisms. The ASV-labile copper concentration gives the best correlations with algal toxicity tests and is considered to be the best measurement of biologically available copper in water (Florence 1992). The relationship between total copper and ASV-labile copper was statistically significant in our experiments, although different for each experiment. This means that copper speciation has to be determined for each investigated water sample and no assumption can be made about the relationship between total copper and its toxic fraction. Furthermore, it may be difficult to compare adverse effects of copper on aquatic organisms reported in the literature because ASV-labile copper levels or other information on copper speciation is usually not provided.

Because of the nature of the experimental conditions, some difficulties were experienced in maintaining constant copper concentrations and water quality during the course of the experiments. Allowance must be made for the variation in copper concentration when interpreting results. Although the constant copper level and constant water quality was not achieved during the experiments, the resulting pulses may better reflect the variability of environmental conditions in Macquarie Harbour to which the farmed fish are exposed.

4.2 Fish survival

Fish survival was affected at concentrations lower than reported in the literature, with most mortality experienced above 20 μgL^{-1} of ASV-labile copper. The reported 96 hour LC50 values for salmonid fish range from 60 to 680 μgL^{-1} of total copper (McKim & Benoit 1971, Wilson 1972, Lorz & McPherson 1976, Dixon & Sprague 1981 a&b, Hansen et al 1996), with the lowest value being for coho salmon, *Oncorhynchus kisutch* (Lorz & McPherson 1976). It is possible that the levels of ASV-labile copper, which are usually not reported in the literature, were lower in these published experiments. The exposure time was longer in our experiments (six to seven weeks) than in a standard toxicity tests (four days). Mortality was experienced after at least two weeks of exposure in our experiments, so it would not have occurred in a routine 96 hour toxicity test. It has been also suggested that laboratory-reared rainbow trout are more resistant to copper than hatchery-reared fish (Hansen et al 1996). Most experiments use laboratory-reared animals, whereas hatchery-reared animals were used in this study. Other factors could affect copper toxicity to fish. Size of fish has a dramatic effect on LC50 results (Howarth & Sprague 1978, Lauren & McDonald 1986). Most LC50 tests use small fish for logistical reasons. The fish used in our experiments were bigger than fish used in previous toxicity tests. In general, copper is more toxic to smaller fish than to large fish, however other confounding effects may result in different relationships between size and LC50. Also, in routine LC50 tests the fish are acclimated to environmental conditions other than the exposure to toxicant, whereas in our experiments the exposure was to mimic transfer to sea cages in Macquarie Harbour so the fish were exposed to greater salinity and copper at the same time. Finally, we used analytical reagent copper sulphate as source for copper and the water contained low levels of organic material and no humic acid which can both bind copper and decrease copper toxicity to trout (Zitko et al 1973, Brown et al 1974).

4.3 Fish growth

In one tank sub-lethal exposure to copper at concentrations averaging 2.7 μgL^{-1} of ASV-labile copper for six weeks significantly reduced growth of rainbow trout, determined as wet weight

gain, but this concentration of copper did not affect the growth of fish in a second tank. Exposure to more than 8 μgL^{-1} or more of ASV-labile copper consistently affected fish growth. Previous studies have demonstrated the effect of copper on fish growth. Weight loss was reported in brown trout and mirror carp exposed for 38 weeks to 290 μgL^{-1} of copper (O'Neill 1981).

Weight gain can have an important influence on biochemical indices in immature fish. RNA concentration and RNA:protein ratio have both been used to indicate rates of protein synthesis and would be expected to decrease with decreasing growth. It is therefore interesting that the RNA:protein ratio showed different patterns in lower and higher exposure concentrations. Several studies have suggested that toxicants have the effect of decreasing growth and protein retention through increased protein degradation (turnover) but not affecting rates of protein synthesis. This may explain the pattern shown by the RNA:protein ratio, in that RNA was less affected by treatment whereas the protein concentration decreased with the increasing copper concentrations. Nevertheless it is possible that a second mechanism, involving increased protein synthesis as well as increased degradation, was in place at the greater copper concentrations. Rates of protein synthesis and RNA activity would also need to be measured to investigate this further.

4.4 Fish health

Copper has been shown to have an adverse effect on fish health and increase fish susceptibility to infections (Dunier 1993). Sub-lethal exposure of chinook salmon and rainbow trout to copper increased their susceptibility to infections by *Vibrio anguillarum* (Baker et al 1983). An increased susceptibility to IHN virus was observed in rainbow trout exposed to sub-lethal concentrations of copper (Hetrick et al 1979). However, other research failed to show any significant effects of exposure to copper on rainbow trout susceptibility to diseases (Carbello et al 1992).

Suppression of phagocytosis by exposure to copper has been documented in the literature. In vitro exposure of carp, *Cyprinus carpio*, leucocytes to $CuCl_2$ at concentrations greater than 6000 μgL^{-1} showed suppressed phagocytosis (Dunier 1993). For rainbow trout, a suppressive effect was observed on the phagocytic uptake of *Staphylococcus aureus* at concentrations from 1000 to 10 000 μgL^{-1} and chemiluminescent response of phagocytes (Elsasser et al 1986).

In vitro exposure to 500 μgL^{-1} of copper stimulated phagocytic activity and NBT (nitroblue tetrazolium) reduction by dab phagocytes from kidney, however it had suppressive effect on dab spleen phagocytes and there was high individual variation (Pulsford et al 1995).

In contrast, no effect could be found in other experiments investigating effects of exposure to copper on phagocytosis. Japanese eel exposed to 100 to 250 μgL^{-1} of copper for 12 hours showed reduced phagocytosis for *Edwardsiella tarda* but not for *Vibrio anguillarum* (Mushiake et al 1985). Copper seems to affect phagocytes function in a complex manner, stimulating or inhibiting the production of immunological intermediates. Chronic exposure to copper may enhance and not suppress activity of phagocytic cells (Muhvich et al 1995).

In our experiments the relative proportion of white blood cells in the control fish was within the range previously reported for rainbow trout (Hoffman & Lommel 1984, Dick & Dixon 1985, Bruno & Munro 1986, Thuvander 1989, Bowser 1993, Thuvander et al 1993, Lamas et al 1994). However, there was a wide variation between different studies and within a study between individual fish. The proportion of circulation lymphocytes was significantly affected by exposure to copper in experiment 2. A similar but much more dramatic trend was shown in rainbow trout after acute or chronic exposure to 301 μgL^{-1} of copper (Dick & Dixon

1985). The same authors also found an increase in circulating thrombocytes after acute exposure but a decrease in the relative proportion of thrombocytes after chronic exposure. No significant difference in the population profiles of white blood cells could be detected in goldfish exposed to 30 to 175 μgL^{-1} of copper for 96 hours (Muhvich et al 1995).

In our experiments there was a trend of reduced antibody production against *Vibrio anguillarum* in the rainbow trout exposed to copper. This is in agreement with other research results. Antibody response to MS2 bacteriophage was suppressed in carp exposed to 290 μgL^{-1} of $CuSO_4$ for 38 weeks (O'Neill 1981). Juvenile coho salmon exposed to 10 to 33 μgL^{-1} of $CuCl_2$ for 1 month showed a suppressed humoral response to *Vibrio anguillarum* (Stevens 1977). The number of antibody-producing cells, as demonstrated by PFC assay, was reduced after 10 days exposure to sections of rainbow trout spleen to 100 to 1000 μgL^{-1} of $CuCl_2$ (Anderson et al 1989). Exposure of blue gouramis to 9 μgL^{-1} of copper for 1 and 3 weeks significantly reduced antibody production (Roales & Perlmutter 1977).

Exposure to copper did not seem to have a great effect on the structure of organs of rainbow trout in our experiment. A wide range of histopathology has been reported in fish exposed to copper, however in most cases the exposure levels were much greater, exposure time much shorter and in some cases the changes were reported only in mechano- and chemoreceptors (Sorensen 1991), not investigated in our study. Fatty metamorphosis was observed in kidney of winter flounder exposed to 560 to 3200 μgL^{-1} of copper for 29 days (Baker 1969). This may be similar to vacuolation of liver cells in fish exposed to copper in our experiment. In contrast to our results, flounder exposed to copper seemed to have reduced number of mucous cells in their gills (Baker 1969). Damage of hematopoietic tissue was reported in winter flounder (Baker 1969), but spleen depletion was noted only in two individuals in our experiments. These differences in the results are possibly due to great difference in exposure levels or water quality between the experiments and species-specific differences.

4.5 Stress and osmoregulation

Exposure to 60 μgL^{-1} or 90 μgL^{-1} of copper (but not 15 μgL^{-1}) for seven days significantly increased cortisol level in coho salmon (Schreck & Lorz 1978). However, cortisol levels declined over 21 days of exposure in rainbow trout exposed to 185 μgL^{-1} of copper (Munoz et al 1991). Our results indicate potential confounding effects of social hierarchy and exposure to copper. The cortisol levels were very variable for individual fish, except for the fish exposed to 19.5 μgL^{-1} of ASV-labile copper (equivalent to 46.8 μgL^{-1} of total copper). Possibly, the exposure to copper inhibited the endocrine system and made the normal response to stress, showed by other fish, impossible. Fish captured from polluted rivers in Canada were unable to elevate cortisol level in response to acute stress and it was suggested that long time exposure to pollutants may lead to an exhaustion of the cortisol-producing endocrine system (Hontela et al 1992). Additionally, exposure to pollutants may not activate the hypothalamo-pituitary-interrenal (HPI) axis in fish.

The fish exposed to the highest levels of copper had elevated sodium and potassium levels in their blood. Although sodium levels may be increased shortly after transfer to sea water, they usually decrease to basal levels within two weeks after transfer (Johnston & Cheverie 1985). In our experiments the levels found in the fish exposed to copper were greater than basal levels for rainbow trout in brackish water (Johnston & Cheverie 1985), indicating osmoregulatory problems. Osmoregulatory problems were reported in channel catfish and golden shiners exposed to 2500 to 5000 μgL^{-1} of copper (Lewis & Lewis 1971) and rainbow trout exposed to 12.5 to 200 μgL^{-1} of copper (Lauren & McDonald 1985) as well as rainbow

trout exposed to 400 μgL^{-1} for 5 hours (Wilson & Taylor 1993). Exposure to copper inhibits branchial Na+, K+, ATPase and thus affects osmoregulation in fish (Lauren & McDonald 1985). Sodium plasma levels and gill lipid metabolism were influenced by exposure of rainbow trout to 100 to 800 mgL^{-1} of copper for four days (Hansen et al 1993). Although it was suggested that these changes happen only within the first seven days of exposure (Lauren & McDonald 1987), it was not the case in our study where the fish had elevated sodium and potassium levels after seven weeks of exposure. This may be due to fluctuations in salinity and other water quality variables and larger fish being used in our experiment, which makes it more applicable to the situation in Macquarie Harbour.

4.6 Copper residue in fish

The liver is responsible for maintaining the internal balance of copper level and hepatic accumulation has been suggested to be time- and concentration-dependent (Sorensen 1991). However, in our study the copper residues in the liver did not seem to be related to the concentrations to which the fish were exposed, as the residue was the greatest in the fish exposed to 8.2 μgL^{-1} of ASV-labile copper and lower in the fish exposed to 21 μgL^{-1} and 19.5 μgL^{-1} of ASV-labile copper. This may be because concentration-dependent copper uptake in rainbow trout does not occur at exposure levels of 9 μgL^{-1} and lower (McKim & Benoit 1974). Additionally time-dependent hepatic accumulation in coho salmon stabilises after 28 to 30 days of exposure (Buckley et al 1982). High individual variation and a lack of difference between exposed and control fish was observed in an experiment with rainbow trout exposed to 2480 to 29400 μgL^{-1} of copper (Carbonell & Tarazona 1993). Furthermore, our histological results showed that the fish exposed to 21 μgL^{-1} and 19.5 μgL^{-1} of ASV-labile copper had a greater increase in mucous cells in their gills and in the thickness of the respiratory epithelium (table 3), suggesting that the fish may have been better protected against copper uptake. Mucus production in response to exposure to copper was reported previously (Lewis & Lewis 1971). About 33% more mucus was secreted by rainbow trout fingerlings exposed to 85 μgL^{-1} of copper than by control fish (Miller & MacKay 1982).

4.7 Implications for fish culture in Macquarie Harbour

Although this study investigated the effects of copper on rainbow trout, the results cannot be easily extrapolated to the situation in Macquarie Harbour because of differences in water quality. In both experiments the fish were exposed to copper only in the form of copper sulphate. The hydrochemistry of Macquarie Harbour is much more complex and although copper is the major obvious contaminant, fish may be also exposed to pH changes, other metals and changes in salinity. On the other hand, Macquarie Harbour water would most likely contain more organic matter and possibly humic acid or other compounds capable of binding copper and making it less available to the fish.

The adverse effects of copper on rainbow trout suggested by the results of this study and published literature indicate that caution should be applied if it is proposed to culture salmonid fish in those sections of Macquarie Harbour most affected by copper pollution. Although some of our results are not statistically significant and sometimes there is a lack of a dose response relationship, they should be treated as potential effects on rainbow trout exposed to copper in brackish water. Rainbow trout tested in this study seemed to be more susceptible to copper, possibly as a result of salinity changes, which are not uncommon in Macquarie Harbour. Not only could the survival of the fish be affected by copper. Sub-lethal concentrations of copper may also reduce growth and lower resistance to infectious diseases or to salinity fluctuations.

The experiments tested the effects of copper only on rainbow trout. It should be noted that no experiments were done with Atlantic salmon, which is the second species cultured in Macquarie Harbour.

4.8 Recommendations for future research

1 Undertake additional copper toxicity studies on rainbow trout and Atlantic salmon investigating potential effects of Macquarie Harbour water on the fish. The study described in this report could not address environmental factors other than copper level and salinity changes. Running the experiments in situ or using Macquarie Harbour water would be an advantage.

2 More research is necessary to determine the best analytical copper speciation method. As the effects detected in our experiments did not always relate to ASV-labile copper or total copper level in water, other measurements of copper speciation, such as free copper ion content in water should be used.

3 Copper levels in Macquarie Harbour should be monitored on a regular basis, particularly in the vicinity of fish farms. Potential use of data loggers for water quality measurements, including copper concentrations, should be investigated.

4 The results of this study did not confirm earlier suggestions that fish acclimate to copper exposure. More research is needed to explain the reasons for the lack of acclimation reported in previous scientific studies.

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