The potential use of metal ratios in the gills of rainbow trout as biomarkers for acute waterborne copper exposure

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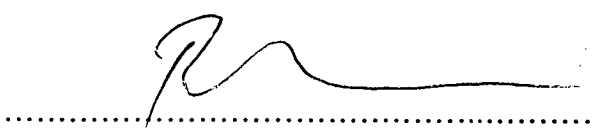

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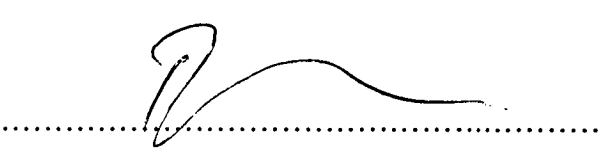

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Abstract

This thesis examined the effects of short-term, acute copper poisoning on the metal concentrations in the gills of rainbow trout (*Oncorhynchus mykiss*) with the aim of developing possible biomarkers under such exposure regimes. The experiments were designed principally to mimic the spillage of high copper contaminants such as industrial or mining wastes into an environment which would flush the contaminant quickly through the water system, resulting in a brief, but acute exposure to copper for the inhabitants of the environment. A variety of water quality conditions were investigated including in fresh and brackish waters, in conjunction with elevated zinc levels in fresh waters and in brackish waters high in dissolved organic carbons.

The use of the gill copper concentrations in a ratio to other metals in the gills was investigated for their potential role as biomarkers for acute copper exposure and in fish kills. The depuration rates of metals from the gills were also examined in the carcasses of animals killed through exposure to elevated levels of dissolved copper in fresh and brackish waters. Data from fish kills in Macquarie Harbour, a large, brackish inlet on the western coast of Tasmania, Australia, historically known for its copper contamination, were included in the thesis.

It was demonstrated that in short-term, acute exposure to copper, hepatic copper levels will not reflect the exposure whereas copper/metal ratios in the gills of rainbow trout may do so. Circulating copper levels in the animal's blood plasma were unaffected. When exposed to mixtures of copper and zinc, the ratios may still be effective indicators, particularly the copper/sodium ratio. Copper residues in the gills

were elevated while sodium levels in the gills and calcium levels in the plasma also decreased significantly indicating an interruption to the animal's ability to iono-regulate.

However in brackish waters copper ratios appear less viable as biomarkers. Altered physiological requirements between the animals in a hypotonic, isotonic and hypertonic ionic environment affected copper accumulation at the gills and the concentrations of other metal in the gills. Metal concentrations in the gills equilibrated to environmental levels in 6 to 45 hours *post-mortem*. It was observed the *post-mortem* depuration of sodium from gill tissue in both fresh and brackish water may provide a means of quantifying the time since death of animals in fish kills. Copper loads in the gills of animals from fish kills in Macquarie Harbour were as high as those of animals killed by copper exposure in laboratory trials in waters of the same salinity, yet the copper/zinc ratios did not indicate that copper exposure was the cause of mortality.

Data was also presented indicating high levels of naturally occurring dissolved organic carbon in brackish waters can have an ameliorative effect on the toxicity of copper to rainbow trout. The concentrations of copper that accumulated in the gills of the exposed rainbow trout decreased as the levels of dissolved organic carbons increased. The concentrations of copper correlated better with the total measured copper in the water column than with the ASV-labile measurements of copper. This indicated ASV-labile copper does not provide a good indicator of the bioavailable fraction of the total measured copper.

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Chapter 1 – General Introduction

Copper in biological systems

Copper is an essential element for all biota. It is a co-factor in more than 30 enzymes (Harris, 1991) including important metallo-enzymes involved in the electron-chain transfer in the mitochondria, scavenging free radicals, bone and connective tissue development and regular cardiac function (Cousins, 1985; Linder and Hazegh - Azam 1996). Two genetically linked diseases related to copper have been identified in mammals, Menke's disease and Wilson's disease, which has sponsored considerable research of copper metabolism in mammals. There is now a considerable body of literature concerning copper uptake, transport and regulation in mammals (for reviews see Camakaris et al., 1999; Cousins, 1985; Harris, 1991).

Copper metabolism is less well understood in fish, although copper toxicity in freshwater systems has been researched for many years (reviewed in Sorensen, 1991). In normal copper metabolism in teleosts the liver is the primary storage organ of the metal and biliary excretion of copper is stimulated when copper uptake increases (Grosell et al., 2001; Westerlund et al., 1998). Copper uptake occurs via the intestine, as in mammals, and via the gills (Grosell and Wood, 2002; Kamunde et al., 2002; Miller et al., 1993). Copper plasma levels generally appear to be tightly regulated.

While this thesis is primarily related to elevated levels of copper in water systems and pollution episodes, it is necessary to recognise copper is a naturally occurring component of fresh and sea water. Ambient concentrations of copper in freshwater systems may range from 0.5 – 5.0 $\mu g\ L^{-1}$, concentrations sufficient to provide a

substantial contribution to the uptake and regulation of copper in its normal homeostasis (Grosell and Wood, 2002). Copper levels in seawater are typically less than 1.0 ug L^{-1} (Turner, 1995).

Copper in society

Copper, as a refined metal, has been used for a variety of purposes in societies for centuries. Its characteristics include high electrical and thermal conductivity, strength and malleability and resistance to corrosion, qualities which make it extremely useful for a broad range of applications. In the modern world copper is used extensively for the production of conductors, electrical cables and transformers, electronic circuitry, computers, water pipes, roofing, heat exchange equipment, plumbing fittings, ship and boat hulls, in automotive and marine motors and fitting, as a basis for coins and also for decorative brassware.

Copper mining and production

Due to the use of copper in industry and society, copper mining and processing has also been widespread. Copper is mined worldwide, major producers being Chile, the U.S.A., China and Australasia. Copper deposits occur primarily as oxides and also as sulphides. The ores are processed into metal either by leaching and electrowinning or by smelting and refining after extraction of the ore deposits.

Since 1900 worldwide production of refined copper has increased from 494 000 tonnes to more than 13 000 000 tonnes in 1997. The demand for copper has increased

steadily throughout this time increasing at a rate of 4.5% between 1945 and 1973 and at an average rate of 2.9% throughout the 1990s (URL: http://mmsd1.nrcans.gc.ca – The International Copper Study Group – 10/01/2004). Prior to the increasing environmental concerns of the last few decades, copper wastes were dumped with little concern for their disposal. For example, the Mt Lyell Copper Mine in south west Tasmania is estimated to have discharged over 90 million tonnes of tailings into the King and Queen River systems over the course of nearly a century of mining (Koehnken, 1996). This has resulted in an enormous copper-rich alluvial fan at the river's discharge point into Macquarie Harbour. Even today copper discharges in river systems may be substantial. In Papua New Guinea, due to the local climatic, geochemical and hydrological conditions, the Ok Tedi copper mine on the upper reaches of the Fly River system has been unable to build suitable retention facilities for the mine tailings. The Government of Papua New Guinea has agreed the mine may discharge fine grain tailings and overburden directly into the river system, the copper concentrations of which are typically 1000 $\mu g\ g^{-1}$ (Apte et al., 1995).

Copper partitioning and speciation in the water column.

Copper in aquatic systems may exist in particulate, colloidal and soluble forms, the soluble forms of copper being the most available to non-vegetative aquatic biota (Stiff, 1971b). Solubility was defined as that fraction of total copper which passed through a 0.45 μm filter, representing a practical compromise between the smallest possible pore size and flow rates. This pore size is now an accepted standard for defining the soluble portion of dissolved metals in toxicological studies (Paquin et al.,

2002) although it is acknowledged some very fine particulate matter may be included in the soluble fraction (Stiff, 1971b).

The major factors affecting copper speciation are pH and complexing ligands (Pagenkopf, 1983; Sylva, 1976; Turner, 1995). At normal pH ranges for aquatic systems the majority of the copper in natural waters is associated with suspended solids. The soluble copper is almost entirely complexed with amino acids, other polypeptides, carbonates and bicarbonates (Stiff, 1971b) or may be removed from the water column by precipitation (Sylva, 1976). Even when the concentration of total copper is high, the processes of hydrolysis, precipitation, complexation and adsorption reduce the free copper concentrations to extremely low values (Sylva, 1976). As pH decreases the levels of free copper increase, until at pH below 5.0 the majority of soluble copper is in the ionic form (Sylva, 1976).

The modelling of metal speciation has developed greatly in the last 20 years. A variety of programs are now available to determine the levels of free and complexed copper in aquatic systems including MINTEQ, MINEQL and WHAM. These programs are dependent on accurate measurements of the water quality parameters and conditional stability constants of the various organic and inorganic ligands within the system. Assuming the system to be in equilibrium an accurate evaluation of the concentrations of the various species of any metal can be determined by simultaneously solving the various equilibrium equations of the reactive species.

Work by Apte et al. (1995) has described the effects of the release of particulate copper from a mine site into a river system. It has been shown that there may be exchanges between the particulate, suspended solid and dissolved phases of copper when high copper loads are released into a river system in the particulate form. These exchange processes have a dual nature: copper may bind to weak ligands within hours, but also to strong ligands at a much slower rate of up to weeks. This demonstrated that under certain conditions of copper release into the environment, the different phases of copper may be in disequilibrium for extended periods of time (Apte et al. 1995).

Effects of copper poisoning on finfish

Copper accumulation in tissues and organs

The liver is considered the primary organ of metal metabolism in teleosts and the primary site of metal accumulation during normal metabolism (Sorensen, 1991). However, hepatic copper levels in unexposed animals may be highly variable between species. For example, Frazier (1984) reported a one-thousand-fold greater concentrations of copper in the livers of white perch, *Morone Americana,* compared to striped bass, *Morone saxatilis,* collected from the same, uncontaminated areas. The concentrations recorded for the white perch were considered an example of 'abnormal' copper metabolism. Table 1.1 shows copper levels in the livers of various fish species at either environmental levels or from control treatments (no added copper) under experimental conditions. Although these data are not directly

comparable because of varying conditions, they indicate the range of copper levels found amongst teleosts.

Table 1.1 Copper levels in the livers of various fish species from uncontaminated sites or unexposed under experimental conditions. (adapted from (Sorensen 1991)

Species	**Exposure time**	**Exposure level** ($\mu g\ L^{-1}$)	**Liver Cu** ($\mu g\ g^{-1}$)	**Reference**
Brook trout	24 mths	3	239	McKim & Benoit, (1974)
Bluegill	24 mths	3	7	Benoit, (1975)
Coho salmon	28 – 30 days	~ 0	70 dw	Buckley *et al*, (1982)
Brown bulhead	20 mths	~ 0	29 dw	Brungs *et al*, (1973)
	30 days	~ 0	23 dw	
Striped bass	Envir.	-	3 ww	Frazier, (1984)
White perch	Envir.	-	2795 ww	Frazier, (1984)

dw = dry weight, ww = wet weight, Envir. = Environmentally exposed.

When animals are exposed to elevated waterborne copper accumulation in the gills is rapid compared to accumulation in the liver (McGeer et al. 2000b). However this accumulation may be followed by a subsequent decrease and then stabilisation of the gill copper burden, indicative of the damage-repair model of acclimation proposed by McDonald and Wood (1993). The binding of copper to the gills is affected by other water quality parameters, particularly pH, hardness and the concentration of dissolved organic carbon (Chakoumakos et al. 1979; Erickson et al. 1996; Playle et al. 1993a). This will be discussed further in the section on copper toxicity.

Miller et al. (1993) found waterborne copper increased accumulation in the livers and kidneys of exposed rainbow trout but not in gills or digesta while dietary copper was found to increase copper accumulation in liver, gill, kidney and digesta. Buckley et al. (1982) found copper accumulation occurred in liver, kidney and gills of coho salmon exposed to waterborne copper with the most significant accumulations occurring in the liver. While some studies have observed copper accumulation in the kidneys, renal copper accumulation and clearance does not appear to be significant compared to hepatic copper accumulation and biliary clearance (Grosell et al. 1998).

In exposures conducted in brackish water, Nowak and Duda (1996) found significant differences in hepatic copper accumulation in rainbow trout between treatments but not all treatments varied from controls. Accumulation may also occur in the kidneys and muscle of seawater adapted flounder (Stagg and Shuttleworth 1982a).

Neither Griffin et al. (1997) nor Perkins et al. (1997) found any accumulation of copper in the muscle of channel catfish exposed to copper. At the intracellular level the majority of copper is found in the cytosol, bound to the metallo-protein metallothionein (Hogstrand and Haux 1991). Table 1.2 summarises copper accumulation in various organs.

Table 1.2 Accumulation of copper in various organs of teleosts after experimental exposures to elevated waterborne copper levels.

Species	[Cu] µg L-1	Liver	Gill	Kidney	Muscle	Blood Plasma	Digesta	Whole body	Reference
Bluegill	12	-	-	+					Benoit
	40	-	+	-					(1975)
	162	+	+	+					
Coho salmon	70	+	+						Buckley et
	140	+	+						al. (1982)
Rainbow trout	30							+	Dixon and
	94							+	Sprague
	194							+	(1981a)
Channel catfish	220	+			-				Griffin et al.
	354	+			-				(1997)
	465	+			-				
Rainbow trout	1.2							-	Marr et al.
	4.6							+	(1996)
	9.0							+	
Rainbow trout	Dietary	+	+	+			+		Miller et al.
	Water-borne	+	-	+			-		(1993)
Rainbow trout	2.8 a, b	-							Nowak and
	8.2 a, b	+							Duda (1996)
	19.5 a, b	-							
Tilapia	50		+			+			Pelgrom et
	200		+			+			al. (1995b)
Channel catfish	326-9.4	+							Schlenk et al. (1999)
Flounder	170 s	+	+	+	+	+	+		Stagg and
	15	+	+	+	+	+	+		Shuttleworth
									(1982a)

All experiments conducted in fresh water unless otherwise noted. a = ASV-labile copper, b = brackish water, s = seawater, + = sig. diff. to control ($P<0.05$), - = not sig. diff. to control. Control concentrations were all between 0 and 5 µg L-1.

Histopathology

Sub-lethal copper exposure may cause damage to numerous tissues and organs, including liver, gills, kidneys, hematopoietic tissues and chemo- and mechanoreceptors. Secondary behavioural effects arising from structural damage to organs and tissues may impair the animal's ability to locate prey, predators or mates, to follow migratory routes or avoid contaminated waters. Histopathological damage arising from copper exposure has been reviewed in Sorensen (1991).

Histological examinations of the gills of fish exposed to acute copper concentrations have shown the collapse and fusing of lamellae, lifting of the epithelial lamellae from pillar cells and the swelling of epithelial cells (Taylor et al. 1996). An increase the number of chloride cells in the gills and a reduction in mucous cells has been recorded (Pelgrom et al. 1995b) as well as the smoothing of apical membranes, swelling of the tubular system and destruction of mitochondria (Sola et al. 1995).

Studies of milkfish fry exposed to 20 and 100 μg L^{-1} for 27 days demonstrated an increase in the number and size of hepatic lysosomes and an enlargement of liver glycogen fields although pathologic alterations were not observed in the study (Segner and Braunbeck 1990). The authors believed the lysosomal response to be specific to increased copper burdens and the glycogen increase to be a general stress response. Other work has shown histopathological alterations in the olfactory epithelium at copper concentration of 40 μg L^{-1} resulting in a loss of olfactory discrimination to odours (Saucier and Astic 1995). Later work demonstrated

morphological changes in the olfactory system that was characteristic of apoptosis (Juiliard et al. 1996).

Physiology

Branchial copper transport involves two main processes. Copper is transported across the apical membrane by both the apical sodium channel and a specific copper transporter (putatively a p-type Cu-ATPase) (Grosell and Wood 2002). Copper within the gill is then transported into the plasma via a Na^+/K^+ - ATPase embedded in the baso-lateral membrane (Grosell et al. 2002). Whereas the baso-lateral transport processes have been recognised for some time, the understanding of the apical transport process is a recent development. Campbell et al. (1999) demonstrated the inhibition of copper uptake by vanadate, a known blocker of p-type ATPases. Copper transport in the mammalian gut is known to be mediated by a p-type ATPase (Linder and Hazegh - Azam 1996). Additionally a putative Cu^{2+}-ATPase has been isolated from fish gills (Grosell and Wood 2002).

Copper disrupts Na^+ transport in freshwater teleosts and is considered the cause of copper toxicity (Lauren and McDonald 1985). Lauren and McDonald (1987b) found 55 $\mu g\ L^{-1}$ of total waterborne copper decreased Na^+/K^+- ATPase in rainbow trout by approximately 33% within 24 hours of exposure. The inhibition was constant throughout the 28 days of exposure until the animals were transferred to clean water initiating a subsequent recovery of enzyme levels. Na^+/K^+- ATPase activity also was inhibited in crude branchial homogenates of tilapia exposed to waterborne copper for

6 days (Pelgrom et al. 1995b). Although all exposure concentrations (50, 100 and 200 μg L^{-1} Cu) showed a decrease in Na^+ /K^+- ATPase activity, results were significant only in the highest treatment concentration. Another study by Beckman and Zaugg (1988) also demonstrated the inhibition of Na^+ /K^+- ATPase activity in Chinook salmon smolt after 18 hours exposure to natural spring waters with elevated levels of copper (48 μg L^{-1} Cu) but not in parr. The authors hypothesised only the enzyme associated with the chloride cells of the smolts was susceptible to inhibition.

Although the inhibition of Na^+ /K^+- ATPase activity has been clearly demonstrated, McGeer et al. (2000a) found a 2.5 fold increase in Na^+ /K^+- ATPase activity in rainbow trout after two months exposure to 75 μg L^{-1} Cu. This indicates the recovery of animals as part of the acclimation process described by McDonald and Wood (1993) and also agreed with the work of Lauren and McDonald (1987b) and Pelgrom et al. (1995b).

The effect of copper on ionoregulation varies between fresh water and brackish or salt water environments. Stagg and Shuttleworth (1982a) exposed flounder to copper in fresh and saltwater conditions. While K^+ plasma concentrations did not vary under either regime, Na^+ and Cl^- increased in freshwater treatments and decreased in saltwater treatments. Similar to Stagg and Shuttleworth (1982a) and in contrast to work on rainbow trout in freshwater Nowak and Duda (1996) found an apparent but not significant increase in Na^+ levels of rainbow trout exposed to copper under brackish conditions.

Several studies have indicated exposure to elevated copper levels in the water column alters the demands for oxygen resulting in physiological adjustments of exposed animals. Such adjustments include increased coughing rates, higher ventilation rates, increased opercular/buccal amplitudes and increased oxygen consumption (Sorensen 1991). However these results are not always consistent. Sellers et al. (1975) found rainbow trout exposed to copper did not show significant changes to the coughing rate, however, ventilation frequency, ventilation amplitude and buccal amplitude were greater than in control fish. The changes in these parameters were greater at intermediate concentrations than higher concentrations of copper. Variations between individual animals were large. Sellers et al. (1975) also noted up to a 25% reduction in arterial oxygen tension at 290 $\mu g\ L^{-1}$ after 86 hours exposure. Wilson and Taylor (1993a) recorded more severe losses of oxygen tension in rainbow trout exposed to copper concentrations of 311 $\mu g\ L^{-1}$ Cu, leading to mortality.

Growth reproduction and larval survival

The effect of copper on the growth, reproduction and survival of fish is variable with species, size, age and water quality. Benoit (1975) found bluegills exposed to 162 $\mu g\ L^{-1}$ Cu for 22 months had reduced survival, retarded growth and inhibited spawning. These parameters were unaffected in fish exposed to 3 (control group), 12, 21, 40 or 77 $\mu g\ L^{-1}$ or less, although larval survival was significantly lower at the lower exposure levels than in controls. No larvae survived at 162 $\mu g\ L^{-1}$.

Seim et al. (1984) exposed steelhead trout embryo and alevin to both intermittent and continuous regimes of copper at a range of concentrations from 3 – 121 μg L^{-1} (water hardness 120 mg L^{-1}). At day 85 post-fertilisation survival of larvae continuously exposed to copper concentrations of 3 –30 μg L^{-1} was 90% or greater. Survival was 74% at 57 μg L^{-1} while animals exposed continuously to concentrations of 121 μg L^{-1} had all died by the day 70 of the trials. Growth of larvae, measured as mean dry weight, was significantly less for animals exposed to 121 μg L^{-1} from day 31 onwards than in the control group. The decrease in weight was accompanied by a reduced feeding by the fish (observational evidence). Significant decreases in weight were recorded after day 45 at 57 μg L^{-1} and after day 63 at 31 μg L^{-1}. No significant differences were found at the lower exposure levels through out the experiment.

In contrast, Marr et al. (1996) found significantly reduced growth of rainbow trout fry exposed to copper levels as low as 4.6 μg L^{-1} in soft water after 20 days. Fish did not recover or return to control growth rates for the duration of the experiment. It was found that whole body copper levels were significantly higher at 4.6 and 9.0 μg L^{-1} than at control (<0.9 μg L^{-1}) and lower exposure levels (1.1 and 2.2 μg L^{-1}).

Work by Buckley et al. (1982)also found coho salmon fry exposed continuously to copper levels of 70 and 140 μg L^{-1} reduced their feed intake and growth subsequently declined in comparison to control groups (no added copper). The affected groups regained appetite and recovered weight gain after 2 and 4 weeks after returning to

uncontaminated waters respectively, although they remained significantly smaller than control animals throughout the experiment.

The majority of studies of the effects of copper on growth and mortality have examined freshwater fish. Nowak and Duda (1996) examined the effects of sub-lethal copper exposure on the growth and health of sea farmed rainbow trout in brackish water (salinity 14.6 and 19.6 ppt). Measurements of wet weight gain, white muscle protein concentration, RNA concentration and RNA:protein ratio showed a general trend towards decreased growth at higher exposure levels.

Sexual differences in mortality and growth were demonstrated in the channel catfish *(Ictalarus punctatus)* exposed to copper levels of 220, 354 and 465 $\mu g\ L^{-1}$ (Perkins et al. 1997). Males suffered higher levels of mortality and reduced growth in comparison to female fish. Although the data were not always significantly different the authors concluded a "decreasing tendency" was apparent. In other studies of differential mortality from to metal toxicity between sexes, the differences may have been due to sexual size dimorphism rather than differences in susceptibility to metal exposure (Tsai and Chang, 1981, cited in. Perkins et al. 1997) While the authors suggest female catfish may have a potential defensive mechanism other than metallothionein expression, they do not expand on this topic. Olsson et al. (1987) noted hepatic metallothionein content increases at the onset of vitellogenesis in female rainbow trout and Fletcher and Fletcher (1980) found zinc bound by vitellin in

female winter flounder. Further studies may provide more information on the sexual differences related to metal exposures.

Copper toxicity in finfish.

The concentration of a substance that will cause 50% mortality to a population of animals over a given time is termed as the LC50 for that period of time. LC50s conventionally have been used to determine the toxicity of a given substance. Age, sex, species, size and water quality may all affect the determination of an LC50 (Sorensen, 1991) limiting the applicability of the parameter across different environmental conditions. However, it has provided a useful guide for the broad determination of the toxic range of a substance. The use of LC50s has declined over time, due to its limitations between different studies, the changing emphasis of research toward sublethal exposures, the advent of modelling of gill/metal interactions and the increase of ethical and animal welfare considerations in research. Table 1.3 lists the 96-hr LC50s for a variety of fish species.

That copper is toxic to aquatic organisms has been recognised for many years. In an early work on aspects of water quality affecting copper toxicity in rainbow trout Brown et al. (1974) cites work by Kellerman, published in 1905 that proposed copper was more toxic in soft, rather than in hard water. Since the late 1960s when water quality criteria was developing as a topical area of environmental regulation and scientific investigation (Sprague 1969) there has been a considerable body of literature produced on aquatic copper toxicity.

Table 1.3 Copper toxicity for total measured copper of various fish species given as 96 hr LC50s

Hardness, temperature and pH are given where possible (adapted from Sorensen, 1991)

Common Name **Species**	**Cu 96 hr LC50** **($\mu g\ L^{-1}$)**	**Hard** **($mg\ L^{-1}$ CaCO3)**	**Temp.** **(°C)**	**pH**
Coho salmon *Oncorhynchus kisutch*	60 – 74	88 – 89	10 – 12	6.8 – 7.5
Blue gourami *Trichogaster trichopterus*	90		26 -28	
Brook trout *Salvelinus fontinalis*	100	45	10.6	7.5
Atlantic salmon *Salmo salar*	125	8 – 10	18 – 21	6.5 – 6.7
Brown bullhead *Ictalurus nebulosus*	170 – 190	202	5 – 25	7.6
Rainbow trout *Oncorhynchus mykiss*	330 250 - 680	374 365	15 10	7.7 8
Fantail darter *Etheostoma flabellare*	330 – 392		20	
Fathead minnow *Pimephales promelas*	430 460 – 490	198 200	16 – 25 20 – 26	7.9 7.5 – 8.2
Johnny darter *Etheostoma nigrum*	483 – 602		20	
Pompano *Trachinotus carolinus*	1970	5468	20 – 25	8.2
Bluegill *Lepomis macrochirus*	1100 2400	45 35	13 – 28 25	7 – 8 7.7

Lauren and McDonald (1985) first proposed copper toxicity to fish in aquatic environments occurred through the inhibition of Na^+ influx, and at higher concentrations the stimulation of Na^+ efflux. Branchial disruption of sodium regulation is now accepted as the toxic mechanism by which copper causes mortality (Grosell et al. 2002; Hollis et al. 1997). Additionally, it is generally accepted that the free ionic form, Cu^{2+}, in the water column is the most toxic species (Campbell 1995), although hydroxide and carbonate complexes may also be toxic (Chakoumakos et al. 1979; Howarth and Sprague 1978; Shaw and Brown 1974).

As water chemistry will affect metal speciation in the water column there have been numerous studies investigating factors modifying copper toxicity. Water hardness, pH and the concentration of dissolved organic carbons have all been demonstrated to affect copper toxicity in aquatic environments (Chakoumakos et al. 1979; Cusimano et al. 1986; Erickson et al. 1996; Lauren and McDonald 1986; McGeer et al. 2002; Meador 1991; Playle et al. 1993a; Playle et al. 1992; Welsh et al. 1993; Zitko et al. 1973). The influence of pH is to increase the concentration of the free copper ion as pH decreases thereby increasing toxicity. Water hardness is known to ameliorate copper toxicity as hardness increases. This is through the competitive interaction of cations for binding sites at the gill epithelium as first indicated by Pagenkopf (1983) in his Gill Surface Interaction Model and later incorporated in the Biotic Ligand Model (Di Toro et al. 2001; Santore et al. 2001). Dissolved organic carbons, composed primarily of tannic and humic acids released from riparian vegetation are long chain acids with numerous cationic binding sites. These acid chains are therefore

able to complex waterborne copper thereby rendering it unavailable to aquatic life forms

As the understanding of the effects of water chemistry on the availability and toxicity of copper to aquatic organisms has increased there has been a shift in the emphasis of toxicity research. Early research attempted to evaluate toxicity on the basis of total measured copper concentrations whereas the focus has now moved to site-specific water quality criteria (reviewed in Paquin et al. 2002). This has led to the development of the Biotic Ligand Model (Di Toro et al. 2001; Santore et al. 2001). The BLM has extended the work of Morel (1983) who first proposed the Free Ion Activity Model (FIAM) which was subsequently reviewed by Campbell (1995) and the GISM of Pagenkopf (1983). The FIAM developed the concept of toxicity being based on the interactions of the free copper ion with cell membranes. The GISM introduced the concept of copper binding to the gills as a function of competitive interactions between cationic species in the water column for binding sites at the gills as well as competition between the gills and various anionic ligands within the water column. Significant work preliminary to the BLM was also produced by MacRae et al. (1999) and Playle et al. (1993b) in deriving conditional stability constants for metal binding to fish gills. The final leg of the BLM is to incorporate chemical speciation modelling developed by Tipping (1994). The BLM is thus able to unify the chemical factors affecting copper availability and toxicity with interaction of the metal at the site of biological activity in aquatic organisms. The model is then able to predict mortality or biological impairment through the proposal of a threshold limit at

which this occurs (Di Toro et al. 2001; Meyer et al. 1999; Paquin et al. 2002; Santore et al. 2001).

Acclimation

Several researchers have noticed that prior exposure to copper will increase a fish's ability to withstand later exposures. McDonald and Wood (1993) defined acclimation as 'an increased tolerance of an elevated, usually lethal, concentration of a toxicant arising from chronic exposure to a sublethal concentration of that toxicant'. Acclimation to a toxicant is thus identified by a decrease in the disturbance of the physiological processes of an animal when challenged at a higher level of toxicant and a corresponding increase in the LC50 of the animal and may be characterised by a damage - repair model (McDonald and Wood 1993). Acclimation to copper has been demonstrated in rainbow trout both in naïve animals in laboratory studies (Dixon and Sprague 1981b; McCarter and Roch 1983; McGeer et al. 2000a) and in animals taken from contaminated sites (Benson and Birge 1985). It would appear, however, that the acclimation is not a permanent effect, and decreases when animals are no longer exposed to sub-lethal chronic level of copper (Benson and Birge 1985; Dixon and Sprague 1981b).

Although an understanding of the mechanism of acclimation is necessary to studies of chronic, sub-lethal exposures of copper, this work deals with short-term, acute exposures. An assumption of the thesis is that animals are naïve to metal exposure at

the time of exposure and acclimation was not included as a factor in these experiments.

Biomarkers

A useful definition of a biomarker is biochemical, physiological, or pathological responses measured in individual organisms which provide information concerning exposures to environmental contaminants and/or sub-lethal effects arising from such exposures (Benson and Di Giulio 1992). While the effects of pollutants may be studied at all levels of biological organisation, from the sub-cellular to the ecosystem (Stegeman et al. 1992), individual responses will precede population or community responses and therefore enable the identification of disturbances at an early stage (Cairns et al. 1987).

To date there is still no reliable biomarker for copper exposure in finfish, either chronic or acute. Gill copper burdens are still considered the most relevant indicator of chronic exposure in freshwater, superior to liver burdens, ion loss, swimming performance or growth indicators (Taylor et al. 2000). However the requisite for control animals makes it difficult to evaluate populations of animals that may have been exposed. Surrogate animals from other areas or laboratories may be used but the complex interactions between water chemistry, metal speciation and gill/metal accumulation make comparisons extremely hazardous.

The use of metal ratios as biomarkers.

Although the use of ratios have the disadvantage of requiring two sets of measurements, and therefore additional time and cost than if a single equivalent assay is performed, ratios have been used previously as biomarkers for pollutant exposure. Mount (1964) found zinc-caused mortality in fish could be identified under laboratory and field conditions by the ratio of the metal in the gill to the operculum, due to the different rates of accumulation of zinc between the two tissues. Nowak et al. (1995) used the ratio of two isomers of the organic pesticide, endosulfan, to demonstrate exposure to the pesticide in the carp, *Cyprinus carpio.* The use of metal ratios in the gills of fish as a means of detecting exposure to elevated waterborne copper was first proposed by Carbonell and Tarazona (1993).

Carbonell and Tarazona (1993) suggested the use of such metal ratios in the gills would be able to detect copper exposure without the use of control animals. Access to control animals may be problematic in fish kill scenarios and in natural waters due to the difficulty of obtaining suitable controls, or surrogate control animals. Carbonell and Tarazona (1991) initially examined farmed rainbow trout, half of which were treated regularly with copper sulphate introduced into the water column as a prophylactic parasite treatment, while the other half of the animals had not been exposed to the copper treatments, to determine if the treatment affected the distribution of metal in various tissues and organs of the animals (Carbonell and Tarazona 1991). The fins, operculum, gill, liver, kidney muscle and bone were all examined for copper, iron and zinc levels. While copper levels were not altered in the

different tissues between treated and non-treated animals, iron and zinc distributions did change between the two groups for all the tissues other than the fins and the operculums. Carbonell and Tarazona (1991) believed these results were due to changes in the production of the metal binding protein metallothionein which would lead to an increase in the iron and zinc levels while copper concentrations would remain unchanged due to increased clearance rates of the metal to maintain homeostasis. In a later publication it was proposed copper/zinc ratios may be a predictor of copper exposure (Carbonell and Tarazona 1993). All unexposed animals had Zn/Cu ratios in the gills of greater than 0.5 while 90% of the animals had ratios higher than 1.5. For exposed animals the ratios were lower than 1.5 for 93% of the animals and lower than 0.5 for 84% of the animals. This suggests that the use of metal ratios in the gills tissue may be worthy of further consideration as biomarkers of copper exposure.

However, both of the cited publications are scarce on details of the experimental procedures and their arguments for metallothionein induction are also weak based on the evidence supplied. No water quality data were provided for the farms from which the animals were taken although it was stated the water quality was checked to ensure that it was free of heavy metal contamination (Carbonell and Tarazona 1991). Only the results of the statistical analyses were presented rather than the levels of the various metals in the tissues and organs (Carbonell and Tarazona 1991; Carbonell and Tarazona 1993). Finally, the dosage and frequency of the treatments was not given other than 'habitually using copper sulphate treatments...at appropriate doses'

(Carbonell and Tarazona 1993). Short-term immersions of between 150 and 500 μg L^{-1}, depending on water hardness, are normal recommended doses (Cross and Needham 1988; Roberts and Shepherd 1986). It was therefore difficult to assess the claims of the authors. However, the concluding comments provided sufficient cause to examine further the use of metal ratios in the gills as biomarkers.

Biomarker research frequently focuses on chronic sublethal exposures, which is a necessary due to the introduction of many anthropogenic stressors into our environment. However, biomarkers for acute exposures are also required, especially in the case of fish kill scenarios. While the responsible toxin for an acute exposure event and consequent fish kill may be identified through a known accident or incident, numerous fish kills regularly arise where no known cause is established (URL: www.epa.qld.gov.au Queensland Government Environmental Protection Agency, 10/01/2004). When fish kills occur there are additional complications in the selection and use of a suitable biomarker due to depuration of the toxin, and the degradation of the carcass, after mortality.

Metallothionein

Numerous studies have demonstrated copper exposure will induce the production of metallothionein, which in turn sequesters copper from other metabolic activities (Dang et al. 1999; Fletcher and Fletcher 1980; Hogstrand and Haux 1990; Hogstrand et al. 1991; Kille et al. 1992). Metallothionein is able to bind copper due to its high frequency of the sulphydryl rich amino acid, cysteine. Metallothionein may have a

potential role as a biomarker of copper exposure. However, large natural variance within populations and the need to know a populations previous history of exposure to metal pollutants limit its applicability (Schlenk 1996).

Mixed metal exposures

Compared to the extensive body of literature and experimental work on single metal exposures, there is a great paucity of studies concerning mixed metal exposures. This may be seen in publications reviewing metal toxicity studies such as Sorensen (1991) where less than 10 papers reviewed laboratory studies of the toxic effects of metal mixtures on teleosts, while nearly 50 papers concerning copper exposure alone were considered. The scarcity of studies on exposures to mixed metals may be considered as surprising given the production of industrial effluents seldom occurs as the release of a single contaminant (Dethloff et al. 1999), and pollution generally is attributable to a combination of toxicants (Cairns et al. 1987).

Studying and assessing the effects of mixtures is complicated by factors additional to those for studies of a single toxicant. The partitioning and persistence of the toxicants in the environment and study animal are likely to be dissimilar, as are the toxic effects upon, and the target organs of the study animal. Interactions between the different toxicants can occur through competition for ligands, binding sites and receptors which in turn can result in complex interactions between biochemical and metabolic pathways. Effects may therefore be additive, synergistic or antagonistic and the ability to discriminate between these interactions may be impossible.

Furthermore, replicating field conditions or comparisons between different sites may be equally difficult (Cairns et al. 1987; Eisler and Gardner 1973; Neal 1987; Sorensen 1991). However, the need for more studies of mixtures of pollutants and toxins is requisite for a better understanding of the impact of our society on the environment.

Studies of mixtures of metal in the environment thus far have looked at either the accumulation of metals in animals from polluted sites (Marr et al. 1995; Woodward et al. 1995), the accumulation of metals from animals in laboratory studies of metal mixtures (Pelgrom et al. 1994; Pelgrom et al. 1995a; Wepener et al. 2001) or physiological effects of mixed metal exposures (Dethloff et al. 1999; Dhanapakiam and Ramasamy 2001; Pelgrom et al. 1997). To the knowledge of the author, there have been no studies to determine possible biomarkers for finfish exposed to mixtures of metals in the environment. This is therefore an area where investigation is required.

Aims

The primary aim of this thesis is to investigate the use of metal ratios in the gills of fish exposed to elevated levels of waterborne copper as potential biomarkers. It was our intention to mimic scenarios where animals are exposed to brief episodes of acute copper poisoning such as may occur through accidents at a mine site or the accidental spillage of industrial wastes or copper-based agricultural treatments. As well as being specific to copper exposure and accurate it was also intended that the method be

simple and utilisable at mine sites in remote areas or by people who are not necessarily biologists or biologically trained.

Chapter 2 Rainbow trout gills are a sensitive biomarker of short-term exposure to waterborne copper

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Abstract

Hepatic copper levels may not indicate short-term exposure to waterborne copper in teleosts. Significantly higher copper loads were found in the gills of rainbow trout exposed to 105 $\mu g \cdot L^{-1}$ total measured copper for a period of 24 hours than in control animals whereas no differences were recorded in hepatic copper levels. A second experiment exposing trout to 153·$\mu g \cdot L^{-1}$ total measured copper also demonstrated significant differences in branchial copper levels between control and exposed animals after 3 hours exposure. The ratio of the copper load in the gills to the liver, and copper/zinc ratios of the gills were also examined. After exposure to 105 $\mu g \cdot L^{-1}$ for 24 hours both gill/liver copper ratios and Cu/Zn ratios in exposed animals were significantly greater than in control animals. After 12 hours exposure to 153 $\mu g \cdot L^{-1}$ total measured copper the gill Cu/Zn ratio was significantly greater than in control animals. These data indicate the gills may be a better indicator of short-term exposure than the liver.

Introduction

While copper is an essential element of biological systems it is toxic at levels not far above those required for normal metabolic activity. In aquatic systems chronic or acute exposure to copper can overcome regular copper homeostasis and cause mortalities in fish populations (Sorensen, 1991). Contamination of aquatic systems may occur as the result of mining activity, acidification of waterways or through the application of therapeutic or remedial agents in agri- and aquaculture industries (e.g. fungicides or parasiticides). Many freshwater systems around the world have elevated copper levels. Correspondingly, there is considerable interest in determining a reliable and robust biomarker for copper exposure in freshwater teleosts, both for monitoring aquatic systems and as an analytical tool for the investigation of fish kills.

When exposed to elevated levels of waterborne copper fish accumulate the metal residues primarily in the liver and also in the gills (Sorensen, 1991). The metal binding protein, metallothionein (MT), is thought to act as a defense mechanism against copper toxicity by sequestering excess copper in the liver. Most recent investigations of copper biomarkers for freshwater teleosts have focused on hepatic copper residues and direct or indirect assays of hepatic MT. These studies have emphasised the role of the liver as the primary site for accumulation of copper and the induction of metallothionein. Previous laboratory studies have shown significant correlation between hepatic copper levels and copper exposure levels (Griffin et al., 1997; Perkins et al., 1997). However, the gills are the primary sites of toxicity, copper

induced mortality in freshwater fish occurring through the disruption of branchial iono-regulation (Lauren and McDonald, 1987a, 1987b).

MT induction may occur in the gills and to a lesser degree, the gut and the kidneys (Hamilton and Mehrle, 1986). However, gill MT induction is highly variable and considered unreliable as a potential bioindicator of copper exposure (Grosell et al., 1997). Metallothionein may be induced by exposure to cadmium, zinc and mercury as well as organic chemicals and other endogenous stress responses. It is therefore necessary to understand an animal's history of exposure to toxicants and other stresses, and caution needs to be exercised in interpreting the significance of MT levels (Schlenk, 1996).

To date few reports have examined copper accumulation after short-term exposure. In short term exposures, accumulation and sequestration of copper by hepatic tissue may not be sufficiently rapid or reliable to indicate exposure. A significant increase in branchial copper, but no difference in hepatic copper levels, was observed in rainbow trout *(Oncorhynchus mykiss)* after two days of exposure to low levels of waterborne copper (14 $\mu g \cdot L^{-1}$; 0.22 $\mu mol \cdot L^{-1}$) (Dethloff et al., 1999). Copper levels in the gills of brown bullheads *(Ictalurus nebulosus)* increased by 88% after exposure to total copper concentrations of 27 $\mu g \cdot L^{-1}$ (0.42 $\mu mol \cdot L^{-1}$) for 6 days whereas hepatic copper levels rose by 37.5% in the same animals (Brungs et al., 1973). Additionally, after a 24 hour period, hepatic copper levels were greater in channel catfish (*Ictalurus punctatus)* initially subjected to 1 hour of confinement stress than in animals exposed

to total copper concentrations of 326 $\mu g \cdot L^{-1}$ (5.13 $\mu mol \cdot L^{-1}$) (Schlenk et al., 1999). It therefore appears as though hepatic copper levels may not provide a sufficiently rigorous test for short term copper exposure.

Furthermore, the impact of circulating copper levels on metal residues in target organs has not been previously examined. Grosell et al., (1997,) found elevated levels of copper in the plasma of rainbow trout after 3 hours exposure but after 24 hours plasma copper levels returned to control values. Significantly, Grosell et al., (1997) determined through the use of radio-tracers, that the elevated plasma copper levels were not explained by newly accumulated copper but were probably due to the rapid mobilization of available and exchangeable pools of internal copper. These temporarily elevated levels of copper in the plasma may be sufficient to impact upon measured residue levels in highly vascularised organs such as the gills and liver. Further work by the authors using single bolus injections of Cu or an infusion method of delivering the toxicant directly to the circulatory system also demonstrated that plasma copper levels are tightly regulated in teleosts and that copper is cleared rapidly and primarily via the liver (Grosell et al., 2001).

An inherent concern when examining fish kill episodes is the lack of control to evaluate analyses of residual toxicants. This is especially relevant to metal studies where large natural variation may occur. Carbonell and Tarazona (1993) have indicated that the use of copper/zinc ratios in the gills may provide evidence for copper exposure without established controls. Both these metals are essential

elements yet are toxic in excess. In an uncontaminated environment animals will regulate copper and zinc levels within a specific range of values and therefore a predictable copper/zinc ratio may be experimentally determined. Under conditions of increased waterborne copper, the accumulation of the excess metal may shift the ratio significantly in favour of the copper, thereby providing evidence of metal intoxication, provided copper accumulation does not affect zinc levels in the gills.

An alternative means to determine recent copper exposure in freshwater fishes may be to analyse the ratio of $gill_{[Cu]}$ to $liver_{[Cu]}$. As copper accumulates more rapidly in the gill than the liver, the ratio between these organs should shift significantly to the gill in the first 2 to 3 days; the period between the initial exposure and the transport of copper to the liver.

The aims of this study were threefold. Firstly to determine if the levels of copper in the gills and livers of rainbow trout show significant differences after short-term exposures (24 hours) to sub-lethal copper levels and to determine thereby if the ratio of $gill_{[Cu]}$:$liver_{[Cu]}$ would shift significantly towards the gill after short-term exposure. Secondly, to determine if short-term copper exposure will affect the levels of zinc in the gills of rainbow trout and then to verify that copper/zinc ratios may be able to indicate recent exposure to elevated levels of waterborne copper in the absence of controls. The final aim of the experiment was to determine if circulating levels of copper in the blood affect copper levels in the gills or liver of rainbow trout.

The gills and liver were chosen because of their importance as the sites of toxicity and accumulation of copper and their highly vascularised nature. The higher level of exposure was chosen to reflect a spillage event in a pristine environment rather than continual exposure through agricultural run-off or the acidification of waterways. The null hypotheses were that there would be no differences in copper residues and copper/zinc ratios of the gills and livers between the treated and control animals.

Methods and Materials

Two experiments were conducted, both of which examined copper /zinc ratios in the gills of control and exposed fish. The first experiment examined gill and hepatic copper accumulation after 24 hours and the effects of perfusion upon the levels of metal residues. The second experiments considered the rate of accumulation in the gills of rainbow trout after 0, 3, 6 and 12 hours.

Experiment 1

Rainbow trout (mean weight 494.9 g, sd 96 g, n = 41) were obtained from the Tasmanian Key Centre for Aquaculture where they were held in 4 000 L re-circulating tanks (pH 6.27, sd = 0.16; T = 17.75 °C, sd = 0.71; Alkalinity 17.29 $mg{\cdot}L^{-1}$ Ca^{++}, sd 2.19, total hardness 38.01 Ca^{++}, sd 10.07; DO 9.2 $mg\ L^{-1}$, sd 0.08; n = 10). The re-circulating systems are supplemented by the town water supply and de-chlorinated by open-air exposure in holding ponds. Animals were fed dry trout pellets (Pivot) at a rate of 1% of their body weight 5 times a week. Fish were randomly allocated to one of four treatments: unexposed and non-perfused, unexposed and perfused, exposed and non-perfused or exposed and perfused.

Exposures to copper were conducted in static 200 l tanks of water from the Key Centre. Four fish were exposed in each trial. Copper was added as $CuSO_4$ and total measured copper concentration was 105 $\mu g{\cdot}L^{-1}$ (sd = 18, n = 10; 1.65 $\mu mol{\cdot}L^{-1}$). Background copper levels in the control tanks were 0.076 $\mu g{\cdot}L^{-1}$ (n = 5, s.d. = 0.005; 0.001 $\mu mol\ L^{-1}$). Each trial lasted for 24 hours. Animals were not fed during the

trials. Nitrate and nitrite levels were monitored before and after experiments. Levels prior to the experiments were undetectable and rose to 0.40 $mg \cdot L^{-1}$ (sd 0.08, n = 5; 8.6 µmol L–1) and to 0.42 $mg \cdot L^{-1}$ (sd 0.08, n = 5; 6.7 µmol L^{-1}) respectively by the completion of the experiments.

After exposure animals were transferred to a 10 l tank and anaesthetised by the addition of 2 – 4 mL of 10% w/v benzocaine in ethanol. The animals for the perfusion treatments were injected with 0.5 ml of ammonium heparin (235 units per ml) and allowed to recover for 5 minutes prior to sacrifice. A catheter was inserted between the second gill bars into the dorsal aorta and a second catheter was inserted into the bulbus arteriosus. The fish were perfused with a copper-free physiologically isotonic salt solution (Wolf, 1963) until the liver colour changed from red to yellow and the gills paled from rich red to a whitish-pink, indicating the loss of blood. Perfusions were performed until no further colour change was noted. After perfusion the animals were dissected, the gills and livers removed and stored at -20°C. Non-perfused animals were sacrificed and the gills and livers removed and stored at -20°C. All metal analyses were performed within a month of the fish being sacrificed.

Experiment 2

Rainbow trout were obtained from Sevrup Trout Hatchery, Bridport, Tasmania and maintained at the National Key Centre in a 4 000 L re-circulating tank Water quality and diet were as for experiment 1. Twenty animals (mean weight = 218.2 g, sd = 44.9 g, n = 20) were used in the experiment.

Twenty animals were transferred to a 400 L flow-through tank (flow rate 360 – 480 L hr^{-1}) and acclimated for 5 days before the experiment. Animals were fed on day 2 and 4 of the acclimation period but not after day 4 or during the exposures. At the commencement of the experiment copper sulphate solution was supplied at a constant rate via peristaltic pumps. Animals were exposed to 153 $\mu g \cdot L^{-1}$ (2.41 $\mu mol\ L^{-1}$) of total measured copper. Background copper levels were as for experiment 1. Five animals were sampled at 0, 3, 6 and 12 hours. Gills were dissected and placed on ice prior to being stored at -20°C.

Metals Analysis

Copper and zinc analyses were performed by a GBC 932 Oxyacetylene Flame Atomic Absorption Spectrometer. Copper and zinc standards were run prior and after each set of analyses to prevent errors due to drift while analysing samples. Samples of gill, liver and feed were spiked with 1 $mg \cdot l^{-1}$ of Cu^{2+} and recovery rates were determined. These ranged from 105 to 113%. Gill samples were also spiked with 2 $mg \cdot l^{-1}$ of Zn^{2+} and recovery rates were found to be between 100 and 102%.

Livers were homogenised by hand with a glass mortar and pestle. Approximately 200 mg of each liver was digested for 1 hour 20 minutes with 5.0 ml of HNO_3 in a MDS-2000 microwave digester (CEM) and made up to 10 ml total volume with deionised water. The anterior gill filaments were dissected from the gill arch and

digested and prepared in the same manner as the liver samples. Gill filament weights were between 50 and 200 mg.

All data presented are for wet weight of tissue (w.w).

Statistical Analysis

Results were analysed by the software package SPSS. A 2-factor orthogonal ANOVA was used in experiment 1, the factors analysed being copper exposure and perfusion. A single factor ANOVA for exposure time was employed in experiment 2. Results were considered significant if $p \leq 0.05$. Homogeneity of variance between groups were verified using Levene's Test. Data were normally distributed as indicated by residual plots other than for the $gill_{[Cu]}$:$liver_{[Cu]}$ ratios where data were normally distributed only following log transformation. *Post hoc* comparisons of means were performed using Tukey's test.

Results.

Experiment 1.

After twenty-four hours of exposure to sub-lethal copper levels, significant differences were found between the levels of copper in the gills of exposed and non-exposed rainbow trout (Table 1: $F = 7.904$, d.f. 1, 40, $p = 0.008$).

Mean residual copper levels in the liver were higher in unexposed animals, but the difference was not statistically significant (Table 1: $F = 3.945$, d.f. 1, 40, $p = 0.054$).

The ratio between $gill_{[Cu]}$ and $liver_{[Cu]}$ demonstrated a significant difference between copper exposed and unexposed animals (Table 1: $F = 6.964$, d.f 3, $p < 0.001$). Although some values for the ratios were outside the normal distribution of values, all data points were seen to be real values and indicative of the high variability seen in tissue metal accumulation.

No difference was found in zinc levels between Cu exposed and unexposed animals for either gill or liver tissue (Table 1: gill: $F = 0.041$, d.f 1, $p = 0.840$; liver $F = 0.022$, d.f. 1, $p = 0.884$). Variations in all groups of animals were large. The lack of significant changes in zinc levels and the increase in copper levels in the gills was reflected in the Cu/Zn ratios of the gills. These were significantly higher in exposed fish than in unexposed fish (Table 1: $F = 7.012$, d.f. 1, 40, $p = 0.012$). No significant differences were observed for the Cu/Zn ratios of the livers (Table 1: $F = 0.922$, d.f. 1, $p = 0.343$).

Copper levels did not differ between the gills or livers of perfused and non-perfused animals. Although the mean value for both gills and livers were lower in the perfused animals the results were not significant (gills, $F = 0.303$, d.f. 1, $p = 0.585$; liver, $F = 0.062$, d.f. 1, $p = 0.804$: Table 1). Results were also non-significant between perfused and non-perfused animals for gill and liver zinc levels (gill, $F = 0.282$, d.f. 1, $p = 0.599$; liver, $F = 0.115$, d.f. 1, $p = 0.737$). No similar trend towards lower values in perfused animals was observed (Table 1).

Experiment 2.

The copper levels in the gills of exposed fish increased significantly from the basal level after 3 hours exposure (Table 2). A further increase was observed after 6 hours exposure, although this was not significantly different from the 3 hour value. No further increase was observed after 12 hours.

Zinc levels of the gills displayed a continual decrease over time, although, none of the observed changes were significant (Table 2).

Mean copper/zinc ratios in the gills were significantly different between groups ($F = 4.882$, df 3, $p = 0.013$) (Table 2).

Table 1.1 Experiment 1; Means and standard deviations for copper and zinc residues, $gill_{[Cu]}/liver_{[Cu]}$ ratios and copper/zinc ratios in the gills and livers of rainbow trout exposed to 105 μgL^{-1} total copper for 24 hours and perfused with physiologically isotonic saline, plus controls. Values other than $gill_{[Cu]}/liver_{[Cu]}$ ratios and Cu/Zn ratios are expressed as μgg^{-1} wet weight of tissue. Significant differences re indicated by *.

	Treatment			
	Control Control (n = 10)	Control Perfused (n = 10)	Cu exposed Control (n =11)	Cu exposed Perfused (n = 10)
[Cu] Gills	2.22 (2.38)	1.64 (1.26)	4.18* (3.40)	3.92* (1.95)
[Zn] Gills	134.05 (34.06)	144.35 (29.20)	140.55 (61.36)	131.48 (71.45)
[Cu] Liver	69.40 (25.10)	62.62 (29.38)	48.30 (31.94)	48.16 (42.21)
[Zn] Liver	25.02 (8.64)	25.87 (15.64)	17.38 (5.53)	16.47 (7.40)
$Gill_{[Cu]}$/Liver $_{[Cu]}$	0.032 (0.030)	0.033 (0.025)	0.178* (0.270)	0.153* (0.154)
[Cu]/[Zn] Gill	0.020 (0.024)	0.011 (0.020)	0.030* (0.020)	0.048* (0.048)
[Cu]/[Zn] Liver	2.852 (0.64)	2.731 (1.12)	2.755 (1.57)	2.712 (1.44)

Table 1.2 Experiment 2; Mean copper and zinc residues, and ratios of copper to zinc in the gills of rainbow trout exposed to 153 μgL^{-1} total copper for 0, 3, 6 and 12 hours. All values other than Cu/Zn ratios are expressed as μgg^{-1} wet weight of tissue. Standard deviations and the range of values are given. Significant differences re indicated by *.

	Treatment			
	0 hours	3 hours	6 hours	12 hours
[Cu] Gills	1.23 (0.29)	9.11* (2.15)	12.72* (6.49)	12.65* (4.70)
[Zn] Gills	136.58 (45.67)	122.07 (53.03)	107.10 (48.83)	74.24 (37.50)
[Cu]/[Zn] Gill	0.011 (0.006)	0.096 (0.063)	0.133 (0.062)	0.228* (0.160)

Discussion

This study demonstrates that copper levels in the gills of freshwater fish may more accurately reflect short-term exposure to elevated copper levels in the water column than metal residues in the liver. These results correspond with two previous investigations that compared branchial and hepatic copper uptake. Dethloff et al. (1999), found copper levels in the gill changed significantly in rainbow trout exposed to 14 $\mu g \cdot L^{-1}$ of copper after 2 days whereas hepatic copper levels were unchanged. Brungs et al. (1973), noted a two and a half fold greater increase in branchial copper residues than in hepatic copper residues after 6 days exposure to 27 $\mu g\ L^{-1}$ copper.

As may be expected from the different copper accumulation rates of the gills and livers, the ratio of $gill_{[Cu]}$:$liver_{[Cu]}$ between exposed and unexposed animals was highly significant. This ratio shows strong potential as a biomarker for short-term copper exposure but is dependent on the high capacity of the gills for the accumulation of copper. If the gill copper load should decrease quickly in the short term after copper exposure, the ratio would no longer be biased towards the gills. Recent work has demonstrated that the copper load in gills may be cleared rapidly once waterborne exposure is terminated although it was not clear if the copper that depurated from the gills had been adsorbed to the epithelial membrane and moved back into the water column, or whether it had been transported across the gill baso-lateral membrane and was cleared via the hepatic copper pool (Grosell et al., 2001). Therefore the depuration rate of copper from the gills requires investigation before copper residues in the gills can be utilised as an indicator of exposure. Similarly

further work must be done on the copper accumulation in the liver to determine the veracity of this method in chronic exposure situations.

The data presented from both experiment 1 & 2 demonstrated a significant difference between Cu/Zn ratios in the gills of exposed and unexposed fish. This difference was detectable after 12 hours of exposure to 153 $\mu g \cdot L^{-1}$ of total waterborne copper. While no statistically significant differences were seen in the gill zinc levels a declining trend was clearly evident. This may be a consequence of copper-zinc interactions whereby copper is able to displace Zn from biologically active molecules such as metallothionein. Evidence for such a displacement is seen in the hierarchy of metal binding affinities for metallothionein, where copper has a greater affinity for metallothioneins than Zn (Hamilton and Merle, 1986). However, data from these experiments are insufficient for further speculation and more research will be required to substantiate or disprove the occurrence of preferential metal binding and displacement within the gills.

Therefore, as zinc levels were apparently unaffected by the short-term exposure to copper, the shift in the ratio is solely due to the increase in copper residues at the gills. However, as copper and zinc are frequently mined in conjunction it will be necessary to elucidate the effect of copper/zinc mixtures on the patterns of tissue accumulation.

That zinc levels were unaffected by the treatments contrasts with Carbonell and Tarazona (1991), who found changes in the zinc levels of gills of farmed rainbow trout that were habitually treated with copper sulfate for remedial purposes. As the frequency and concentrations of farm treatments were not presented by Carbonell and Tarazona (1991), direct comparisons of data were not possible. Neither were copper residue values for the gills given. However remedial treatments are frequently at high dosages of copper and may be repeated over time rather than as a single acute exposure. Further investigations into the gill ratios of copper and zinc under range of exposure concentrations and conditions (e.g. various water hardness and pH) will clarify whether this technique will be applicable as a control method in the absence of experimental controls.

Circulating levels of copper did not have a significant effect upon the copper load of the highly vascularised organs, the gills and liver. While the mean values for perfused animals were lower than in non-perfused animals the large variances of the data were sufficient to obscure any significant findings. As high variability is common in metal residue studies it may be safe to conclude that circulating copper levels do not need to be considered in future studies of copper tissue residues.

This experiment also underlines the importance of sampling gills as a standard procedure when investigating fish kills or monitoring aquatic pollutants. The use of appropriate and relevant sampling is essential in both toxicological studies and environmental monitoring to obtain meaningful data (Nowak, 1997). The gills are the

primary interface between the environment and the animals internal milieu and are an important site of accumulation for many transition metals (Sorensen, 1991) and also many organic pollutants (Landrum et al., 1996). For example, endosulfan exposure in *Cyprinus carpio* in cotton growing regions of New South Wales was detected by examining gill residues (Nowak et al. 1995). In *Heteropneustes fossilis* exposed to sub-lethal levels of malathion for 10 days greater residual accumulation was found in the gills than in the ovaries, kidneys, liver or muscle (Dutta et al., 1994). Trifluralin was found to cause more severe changes to the biochemical function and histology of the gills than the liver (Poleksic and Karan, 1999). Finally, a significant accumulation of organophosphates was found in the gills of juvenile *Leiostomus xanthurus* that had been exposed to contaminated sediments whereas no residues were found in the livers (DiPinto, 1996).

In conclusion, the gills appear to be a sensitive indicator of short-term copper exposure and a more robust indicator of copper exposure than the liver under such conditions. Both $gill_{[Cu]}/liver_{[Cu]}$ ratios and gill copper/zinc ratios demonstrated significant differences between exposed and control animals and may provide a biomarker for copper exposure. However these hypotheses require further investigation under a broad range of environmental conditions and require validation in field studies. Additionally, circulating copper levels in exposed fish did not impact on the residual metal levels of the target organs examined and need not be considered in subsequent bio-marker research.

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Chapter 3 Copper/metal ratios in the gills of rainbow trout (Oncorhynchus mykiss) provide evidence of copper exposure under conditions of mixed metal exposure

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Abstract

Previous work has suggested the ratio of copper residues to zinc in the gills of rainbow trout may indicate short-term exposure to elevated levels of waterborne copper. However the effect of exposure to a combination of elevated copper and zinc concentrates in the water column was unknown. Rainbow trout were exposed to 8 ± 2 μgL^{-1}, 40 ± 2 μgL^{-1} and 90 ± 9 μgL^{-1} of waterborne copper and 21 ± 3 μgL^{-1}, 129 ± 40 μgL^{-1} and 202 ± 40 μgL^{-1} of waterborne zinc in a two-factor experiment and gill copper and zinc residues were examined. Other gill parameters analysed included the concentrations of calcium, magnesium, sodium and potassium while liver copper and zinc concentrations and plasma copper, calcium, sodium and potassium were also reported.

Copper residues in the gill filaments were significantly higher in the highest level of copper exposure (High Cu, 4.06 μgg^{-1}; Low Cu 2.41 μgg^{-1}; Bkgrnd Cu 2.01 μgg^{-1}; $p = 0.001$) whereas no differences were seen in zinc concentrations at any treatment level. Gill sodium and plasma calcium concentrations were also depressed at the highest waterborne copper concentrations.

While copper/zinc ratios in the gills were significantly different between the highest and lowest copper treatments ($p = 0.002$, $F = 6.59$), copper/sodium and copper/magnesium ratios were more sensitive to waterborne copper exposure ($p = 0.001$, $F = 17.91$ and $p = 0.002$, $F = 15.45$ respectively). These copper/metal ratios may be better indicators of copper loading in the water column.

Introduction

While an essential element for all biota, dissolved copper in the water column reach concentrations that are toxic to aquatic organisms (Eisler, 1997; Sorensen, 1991; Taylor et al., 1996). Copper pollution in freshwater systems, due to mining activities, industrial effluents, agricultural and aquacultural practices and acidification of freshwater systems is now a ubiquitous problem (Carbonell and Tarazona, 1991; Eisler, 1997; Eriksen et al., 2001; Shen et al., 1998; Wepener et al., 2001; Woodward et al. 1995). Despite the extensive research of piscine metal toxicology in freshwater systems (see (Paquin et al., 2002)), a simple and reliable biomarker for copper exposure in fish is yet to be found either for the investigation of fish kills or to monitor metal pollution in aquatic waterways (Taylor et al., 2000).

Mining activity may occur in remote areas and developing nations. Examples are the Marcopper copper mine in the province of Marinduque, in the southern Philippines and the Ok Tedi mine in western Papua New Guinea. At such sites technology may be available for mineral, but not biological, analyses. Also, in such remote regions there are additional complications and costs in the preservation of biological specimens for transport to laboratories suitable for further analysis. As such, a simple and reliable method for assessing the possible exposure of aquatic animals to waterborne contaminants using the facilities available on site is required. Metal ratios in gill tissue are a simple assay method using equipment that is normally available at a large mining site.

A problem facing investigators of fish kills and the effects of pollution events such as mine spills is a lack of controls for the evaluation of animals suspected of being exposed to elevated ambient levels of toxicants. This problem is exacerbated in the case of metals such as copper where naturally occurring levels of variation may be high. Carbonell and Tarazona (1993) and Daglish and Nowak (2002) have suggested a ratio of metal concentration in the gills may circumvent this problem. As the levels of these metals are regulated for normal homeostasis it was proposed that a predictable ratio of metals in the gill tissue may be determined under non-contaminated conditions. When a pollution events occurs the concentration of the toxicant may increase in the gill tissue without a concurrent increase in the secondary element. The ratio will then shift to favour the toxicant, indicating recent exposure to the toxicant. Earlier work validated the premise that copper/zinc ratios in the gill tissues of rainbow trout can indicate a recent exposure to waterborne copper (Daglish and Nowak, 2002).

Copper may also affect the levels of other metals in gill tissue (Carbonell 1993). Metal ratios between gill and opercular tissue have been shown to indicate exposure to zinc in freshwater fish (Mount 1964). However, copper and zinc are often mined in conjunction and spillage from mines or seepage from a tailings dam may contain a mixture of copper, zinc and other metals. It was therefore decided to investigate the effect of mixed metal exposure on the copper/zinc ratios of the gills as well as the ratios of metals other than zinc to copper in the gills as possible biomarkers for copper exposure.

Other than zinc, candidate metals chosen for analysis of ratios against copper as potential biomarkers were sodium, calcium, magnesium and potassium. These metals are all essential elements and their levels within teleosts are tightly regulated. Calcium and magnesium are also important ions in freshwater systems in determining water hardness. Increased water hardness has long been known to ameliorate the effects of metal toxicity in aquatic systems, (Lauren and McDonald, 1986; Miller and Mackay, 1980; Playle et al., 1992) although recent research has discriminated between the importance of the different ions (Welsh et al., 2000).

In freshwater fish the mechanism of copper toxicity is through the disruption of branchial iono-regulation (Lauren and McDonald, 1987a; Lauren and McDonald, 1987b). Lauren and McDonald (1985) demonstrated copper toxicity occurs in freshwater fish through two processes. Firstly, there is the inhibition of sodium influx due to copper's affinity for transport enzymes in the gills epithelium, particularly Na^+ K^+-ATPase. The secondary mechanism by which copper toxicity occurs is through an increase in the passive efflux of sodium, presumably due to a loss of integrity of the intercellular tight junctions and increase in permeability of the branchial epithelium. Potassium efflux was also shown to be stimulated by copper exposure although the copper-dependent potassium losses were independent of the sodium as shown by the plasma K^+/Na^+ ratio. In control fish potassium losses closely equated with the predicted ratio. A possible synergistic effect between copper and low pH was also

implied by the authors. Sodium and potassium were thus seen as potential candidate metals in the investigation of copper metal ratios as biomarkers of exposure.

Calcium is well documented as ameliorating the toxic effects of dissolved metals, predominantly via competitive inhibition at the available cation-binding sites on the gills (Pagenkopf, 1983). Calcium also helps maintain the integrity of the intercellular tight junctions within the cells preserving cellular integrity in freshwater. Some metals, notably zinc, cadmium, manganese and lead, behave as metabolic analogues of calcium and their uptake occurs via the calcium channels in the gill epithelium (Markich and Jeffree,1994). Copper does not share the 'metabolic analogue' characteristics of these transition metals (Wood, 1992). However in the light of Pagenkopf's work on competitive equilibrium at the gill surface, and the Biotic Ligand Model of gill-metal interaction (Di Toro et al., 2001; Santore et al., 2001) copper potentially may displace calcium from biological ligands at the gill surface and thus compromise the epithelial integrity and ion regulation at the gill/water interface.

Magnesium apparently does not have the same level of ameliorative effect upon metal toxicity as calcium (Welsh et al., 2000). In aquatic vertebrates magnesium uptake occurs primarily through the gut while the gills provide a secondary uptake pathway and internal regulation occurs primarily at the kidneys (Bijvelds et al., 1998). However, as an essential element and one of the constituents of water hardness, gill magnesium was considered as a potential biomarker for copper toxicity.

The main aims of this work were; to confirm that the Cu/Zn ratios in the gills of rainbow trout would be an effective predictor of short-term copper exposure in a mixture of waterborne copper and zinc under laboratory conditions, to determine if the ratios of copper to the other essential elements (Ca, Mg, Na and K) could also be used as predictors of copper exposure under the same conditions and to examine the effects of the waterborne metal mixture on metal concentrations in the gill tissue and plasma of freshwater teleosts.

Methods and materials

Animals

Rainbow trout (n= 88; mean wt 241.4g; SD = 50.3g) were obtained from the Aquaculture Centre, University of Tasmania. The animals were held in a 4 000 L recirculation tank supplied with town water supplemented by the addition of $CaCl_2$ and $MgSO_4$ to increase the total hardness. Animals were transferred to 300 L tanks of the same water quality for the experiments. Water quality parameters, given as mean and standard deviation, were T = 13.2 ± 0.2 C°, pH = 7.6 ± 0.1, D.O. = 8.9 ± 0.4 mgL^{-1}, total NH_3 (before) = 0.4 ± 0.4 mgL^{-1}, total NH_3 (after) = 1.4 ± 1.4 mgL^{-1}, Ca = 18.1 ± 0.1 mgL^{-1}, Mg = 14.4 ± 5.4 mgL^{-1} and total hardness = 102.7 ± 2.5 mgL^{-1} as $CaCO_3$; n = 27.

Experimental Design

A two-factor orthogonal design was used with three copper concentrations (8 ± 2 μgL^{-1}, 40 ± 2 μgL^{-1} and 90 ± 9 μgL^{-1}; n = 18) and three zinc concentrations (21 ± 3 μgL^{-1}, 129 ± 40 μgL^{-1} and 202 ± 40 μgL^{-1}; n = 18) giving a total of nine treatments. Copper and zinc were added as sulphate salts. Total additions for low and high copper and low and high zinc were approximately 80 mg and 160 mg of $CuSO_4.5H_2O$ in 300L and 87 mg and 174 mg of $ZnSO_4.7H_2O$ in 300L respectively. Water from each treatment was sampled in triplicate before and after the experiment. The experiment was replicated three times. Three to four fish were used in each replicate. Fish were randomly assigned to tanks and tanks were randomly assigned a treatment

in each replicate of the experiment. All tanks were washed with 5% HNO_3 and rinsed with freshwater prior to the start of the trials.

After 12 hours exposure animals were transferred to a 10 L tank and anaesthetised by the addition of 2 - 4 mL of 10% w/v benzocaine in ethanol. A 1- 3 mL blood sample was taken by caudal puncture before the animals were sacrificed by further exposure to the benzocaine solution. Animals were then weighed and sampled. Samples of gill and liver tissue were also taken and kept on ice prior to being stored at -20°C.

Metals Analysis

Anterior gill filaments (50 – 200mg) were dissected from the gill arch and digested in 5 mL of concentrated HNO_3 in Teflon digest vessels in a CEM MDS-2000 microwave digester (2.5 minutes 630 W; 15 minutes 500 W; 15 minutes 500 W). Approximately 200 mg of liver tissue were homogenised by hand using a glass mortar and pestle and digested in a mixture of 4.5 ml of concentrated HNO_3 and 1.5 ml of concentrated H_2SO_4 under the same microwave conditions as gill samples. Digest vessels and glassware were washed in 10% HNO_3 prior to use. Plasma samples were diluted with deionised water at a ratio of 1:100 for calcium and potassium analysis and 1:500 for sodium analysis. Plasma samples for copper and zinc analysis were diluted at a ratio of 1:1 with a 10% v/v solution of Triton X-100 in deionised water and digested overnight at 35°C.

Metal analysis was performed using a Varian Spectra AA 300 Atomic Absorption Spectrometer (AAS). Calcium analysis was performed using a nitrous oxide/acetylene flame. Gill and liver copper, gill and liver zinc and magnesium and potassium samples were analysed using an air/acetylene flame. Sodium analysis was performed by emission spectrometry. Plasma copper and zinc were analysed by Varian GTA – 96 graphite furnace AAS. National Institute of Standards and Technology bovine liver samples (NIST 1577b) were run to verify the laboratory methods. Standards were run prior and after each set of analyses to prevent errors due to drift while analysing samples. Samples of gill and liver were spiked with 1 mgL^{-1} of Cu^{2+} and recovery rates were determined. These ranged from 100% to 110%. Gill samples were also spiked with 2 mgL^{-1} of Zn^{2+} and recovery rates were between 103% and 108%.

All data presented are for wet weight (w/w) of tissue.

Statistical Analysis

Statistical analyses were performed using the software SPSS v.10.0. Normality of all variables was confirmed using P-P plots, whereby the cumulative proportion for the variable is plotted against the expected cumulative proportion if the data is normally distributed, and histograms.

All the metal levels in the gill tissue were transformed into a ratio using the formula $Log_{10}((Cu/M)*1000)$, where M is the metal of interest other than copper. The

multiplier of 1000 is used to increase the magnitudes of the ratios to simplify further analysis. The ratios were log transformed to satisfy the assumption of equality of variance between groups.

Univariate ANOVAs were performed on gill metal residues with treatment as the fixed factor and the various Cu/M ratios as the dependent variable. A second two factor, univariate ANOVA was also performed, the factors being Cu treatment and Zn treatment. Tukey's test was used for post hoc comparisons of groups.

The gill data from the three experiments were pooled and analysed using MANOVA. For the plasma analysis, only data from the third experiment were used due to incomplete data sets in the first two trials. The independent variables for all multivariate analyses were Cu treatment and Zn treatment. For the gill data set the dependent variables were: gill Cu, gill Zn, gill Ca, gill Mg, gill Na and gill K. For the plasma data set the dependent variables were: plasma Cu, plasma Ca, plasma Na and plasma K. Differences between treatments were considered significant at the level $\alpha = 0.05$ using Pillai's Trace. Correlations between dependent variables were checked prior to the analyses being performed. *Post hoc* tests were performed using Scheffe's method.

Liver data were analysed using a two factor ANOVA, the independent variables being Cu treatment and Zn treatment. The dependent variables were liver Cu and liver Zn.

Results

Fish

All animals survived all treatments. For plasma analysis a complete data set was obtained only for the last replicate of the experiment giving a final sample size of n = 36 with 4 replicates from each treatment.

Gill Metal Levels

Using MANOVA, a significant difference was found between Cu treatments for the gill data set (Pillai's Trace = 0.375, F = 2.888, d.f. = 12, p = 0.001, n = 88). Using Scheffe's Method for *post hoc* comparisons, gill Cu concentrations were significantly higher in the high copper treatment than either the low or background groups (Fig. 3.1, Table 3.1) and Na levels of the gill tissue were significantly depressed in the high copper treatment when compared to the control group (Fig 3.2, Table 3.1). No other metals demonstrated a significant shift in their levels in the gill tissue. Multivariate analysis did not discriminate between any groups for the zinc treatments.

Plasma Metal Levels

Plasma Ca levels of animals in the high copper treatment were significantly depressed (Pillai's Trace = 0.267, F = 3.392, d.f. = 6, p = 0.004, n = 36) compared to both the low and background copper groups (Table 3.2). No significant differences were recorded for any plasma ions in the Zn treatments.

Liver Metal Levels

No significant differences were recorded for either hepatic copper or zinc at any treatment level.

Table 3.1. Means and standard deviations for metal levels in the gills of rainbow trout exposed to waterborne Cu/Zn mixtures. Significantly different results between the low, high and background copper treatments, according to Pillai's Trace are indicated by ^{a} or ^{b}.

	Treatment		
	Bkgrnd Cu (n = 29)	Low Cu (n = 28)	High Cu (n = 31)
Gill Cu ($\mu g g^{-1}$)	$2.01^{a} \pm 0.80$	$2.41^{a} \pm 0.99$	$4.06^{b} \pm 3.63$
Gill Zn ($\mu g g^{-1}$)	261 ± 175	201 ± 125	210 ± 100
Gill Ca ($\mu g g^{-1}$)	2080 ± 1060	2162 ± 1340	2288 ± 1190
Gill Mg ($\mu g g^{-1}$)	351 ± 111	344 ± 130	356 ± 138
Gill K ($\mu g g^{-1}$)	1513 ± 736	1705 ± 560	1516 ± 750
Gill Na ($\mu g g^{-1}$)	$1122.9^{a} \pm 247.1$	$1020.3^{ab} \pm 257.5$	$908.6^{b} \pm 266.3$

Table 3.2 Means and standard deviations for metal ion concentrations in the plasma of rainbow trout exposed to waterborne Cu/Zn mixtures. Significantly different results between the low, high and background copper treatments, according to Pillai's Trace are indicated by [a] or [b].

	Treatment		
	Bkgrnd Cu (n = 11)	Low Cu (n = 12)	High Cu (n = 12)
Plasma Cu ($ngmL^{-1}$)	691.0 ± 90.7	756.0 ± 61.1	699.9 ± 94.3
Plasma Ca (μgmL^{-1})	76.67^{a} ± 14.69	73.16^{a} ± 8.88	61.05^{b} ± 9.01
Plasma Na (μgmL^{-1})	2457.0 ± 211.0	2498.5 ± 290.3	2438.4 ± 283.9
Plasma K (μgmL^{-1})	117.1 ± 36.9	115.1 ± 21.4	120.3 ± 27.7

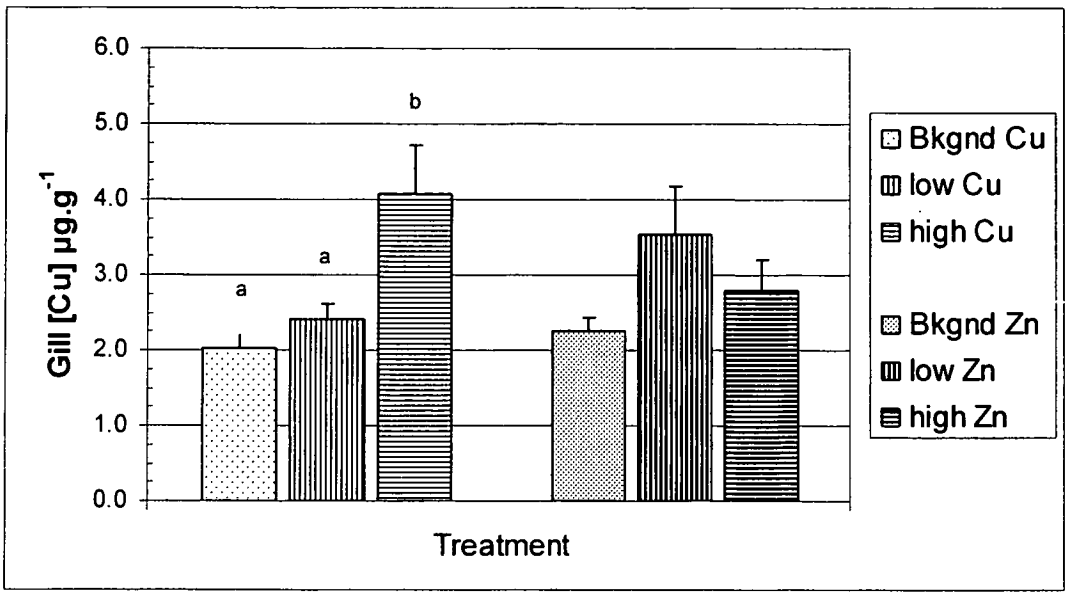

Fig 3.1 Mean gill Cu concentrations and standard errors of rainbow trout exposed to waterborne mixtures of copper and zinc. Data were analysed by univariate ANOVA of both Cu treatment and Zn treatment. Bars not sharing the same letter are significantly different.

Gill Cu/metal ratios

Two different methods of analysing the Cu/M ratios were employed. Firstly, the transformed ratios were analysed by a two-factor, univariate ANOVA with Cu treatment and Zn treatment as the fixed factors. For the copper treatment, significant differences ($p < 0.05$) were found for all the Cu/M ratios. While Cu/Zn ratios were significantly different ($F = 6.593$, $p = 0.002$, d.f. = 2, $n = 88$), the most sensitive descriptors were Cu/Na ($F = 6.593$, $p = 0.002$, d.f. = 2, $n = 88$) and Cu/Mg ($F = 6.593$, $p = 0.002$, d.f. = 2, $n = 88$), as can be seen by the high F-values and corresponding low p-values (Table 3.3). The Cu/Na and Cu/Mg ratios were also significantly different between treatment levels for Zn treatments ($n = 88$; Cu/Na $F= 4.024$, $p = 0.22$, d.f. = 2; Cu/Mg, $F = 6.505$, $p = 0.002$, d.f. = 2). There were no significant effects for interactions between the Cu and Zn treatments.

Secondly, the transformed ratios were analysed by a single factor univariate ANOVA, where 'treatment' was the fixed factor. In this analysis Cu/Na ratios (Fig 2) and Cu/Mg ratios were significantly affected ($n = 88$; Cu/Na; $F = 3.550$, $p < 0.001$, d.f.= 8, Fig 3.2: Cu/Mg $F = 3.342$, $p = 0.002$, d.f. = 8, Fig 3.3) however, no other ratios showed significant differences.

Table 3.3 Unvariate two-factor ANOVA of gill Cu/M ratios of rainbow trout exposed to waterborne mixtures of copper and zinc with copper and zinc as the fixed factors. All results are for the 'copper' treatment.

Cu/M	d.f.	F value	p-value
Cu/Zn	2	6.593	0.002
Cu/Ca	2	3.906	0.024
Cu/Mg	2	15.451	<0.001
Cu/Na	2	17.909	< 0.001
Cu/K	2	4.623	0.013

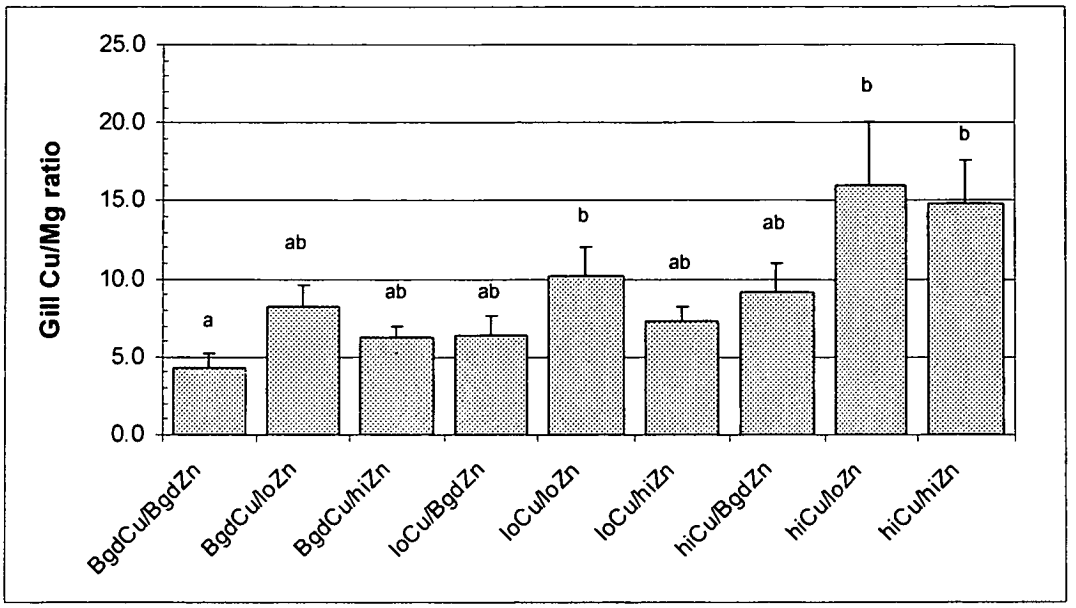

Fig 3.2 Mean gill Cu/Na ratios and standard errors of rainbow trout exposed to waterborne mixtures of copper and zinc. Bars not sharing the same letter are significantly different. The treatments, from the left, are; Bkgnd Cu/Bkgnd Zn, Bkgnd Cu/Low Zn, Bkgnd Cu/High Zn, Low Cu/ Bkgnd Zn, Low Cu/Low Zn, Low Cu/High Zn, High Cu/ Bkgnd Zn, High Cu/Low Zn, High Cu/High Zn.

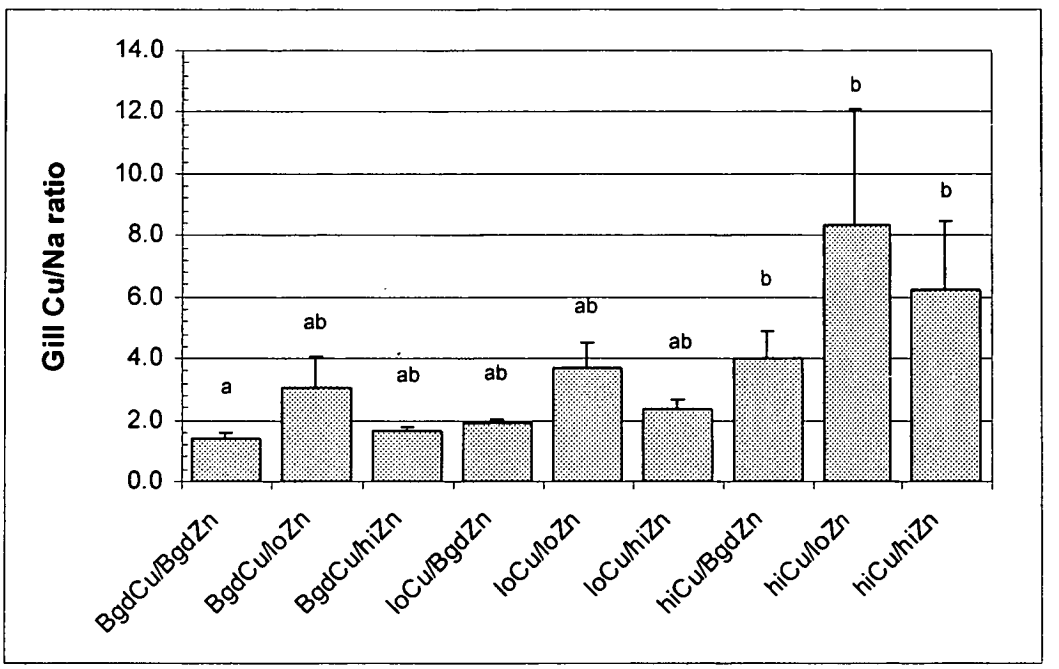

Fig 3.3 Mean and standard errors of gill Cu/Mg ratios in rainbow trout exposed to waterborne mixtures of copper and zinc. Significant differences between treatments are indicated by 'a' or 'b'. The treatments, from the left, are; Bkgnd Cu/ Bkgnd Zn, Bkgnd Cu/Low Zn, Bkgnd Cu/High Zn, Low Cu/ Bkgnd Zn, Low Cu/Low Zn, Low Cu/High Zn, High Cu/ Bkgnd Zn, High Cu/Low Zn, High Cu/High Zn.

Discussion

This work demonstrates that the concentrations of copper in the gills of rainbow trout increase for animals exposed to a waterborne mixture of copper and zinc while the concentrations of zinc, calcium, magnesium and potassium were not significantly altered. Gill sodium concentrations decreased under these exposure conditions. Plasma concentrations of copper, sodium, and potassium were not significantly different between treatments while plasma calcium levels were seen to decrease significantly. There was no effect upon copper or zinc concentrations in the liver.

The lack of change in patterns of total measured zinc in the gills is in accordance with previously published works (Alsop and Wood, 2000; Alsop et al., 1999). However, Alsop and Wood, (2000) and Alsop et al., (1999) were able to determine changes in newly accumulated Zn by using radio-isotope tracers, which was beyond the scope of this work.

The depression of gill sodium levels is not surprising as copper is known to inhibit branchial sodium uptake (Grosell and Wood, 2002). However, the authors believe this is the first documented evidence of the disruption of branchial iono-regulation affecting the levels of sodium in the gill filament tissue rather than the plasma sodium levels. In this experiment plasma sodium was not affected by either copper or zinc treatments (Table 3.2). As plasma sodium levels were not significantly different between treatments, it would seem unlikely the change in tissue levels is a result of residual blood within the gills but is a true indication of tissue sodium levels. Lauren

and McDonald, (1985) suggested the disruption of branchial sodium may be due to a loss of integrity at the inter-cellular tight junctions and a consequent loss of ions down the concentration gradient. An alternative explanation is that waterborne copper is competing with sodium to be transported across the epithelial membrane. While the Cu^{2+} and Na^{+} ions have different physical and chemical characteristics, it is known that gill Na^{+}- K^{+}-ATPase activity is compromised by copper exposure. (Li et al., 1998) It therefore appears credible that a similar mechanism may be affecting rainbow trout.

Due to the depression of sodium levels in the gill tissue, and the concurrent increase in gill copper levels it was considered relevant to investigate the use of the Cu/Na ratios in the gills as a biomarker. As was expected, these ratios also showed a significant difference between the highest copper treatments and background treatments when analysed by univariate ANOVA using 'treatment' as the fixed factor (Fig 3.3). While the Cu/Na ratios appear to be more sensitive than the Cu/Zn ratio as indicated by the F- ratios (Table 3.3), there was no difference between the lower copper treatment and the background treatments. However, the Cu/Na ratios were significantly greater for all the combinations of the high copper treatment with the various zinc treatments.

Cu/Mg ratios were also significantly different between Cu treatments but when analysed by univariate ANOVA using the different treatment regimes, only two of the high copper combinations were significantly different from the other treatments.

These data however, are encouraging for the development of copper specific biomarkers based on gill metal ratios and require further investigation, especially under field conditions

The Cu/Zn ratios obtained for animals in the highest copper treatment were significantly different to the background treatments but no difference was found between the low copper treatment and background animals. This finding is important for further work investigating gill Cu/Zn ratios as a biomarker where animals may have been exposed to a mixture of both metals, e.g. in spillage events from mines where ores contain both copper and zinc. It also validates previous work by (Daglish and Nowak, 2002) utilising gill Cu/Zn ratios as biomarkers of copper exposure. However, it also demonstrates that the ratio is only effective when animals have been exposed to high ambient levels of copper, in this experiments 90 μgL^{-1} of total measured copper. While this may be a realistic concentration in mine spills or industrial accidents it may be greater than the levels of exposure that are normally considered environmentally relevant.

Interestingly, plasma calcium was also depressed in the high copper treatment, but was unaffected by the zinc treatments. Zinc is a known blocker of calcium channels and is considered a 'metabolic analogue' of calcium (Markich and Jeffree, 1994) indicating zinc is transported across the epithelium via calcium channels, and can therefore interfere with the normal regulation of internal calcium levels. No effect was found on the levels of calcium in the gill tissue for either zinc or copper

treatments (Table 3.1), which may suggest that intracellular calcium levels were unaffected. A similar depressive effect on plasma calcium has been demonstrated in *Oreochromis mossambicus* exposed to mixtures of waterborne copper and cadmium (Pelgrom et al., 1997). Cadmium is also a known metabolic analogue of calcium (Markich and Jeffree, 1994). This finding may be explained by a two-fold mechanism whereby a metabolic analogue of calcium (in this case, zinc) may block calcium channels while the additive affect of copper, in competitive equilibrium with Ca for binding sites at the gill epithelium, is sufficient to compromise cellular integrity when the levels of the calcium analogue alone are insufficient to do so.

In conclusion, this work shows that while copper concentrations in gill tissue increase under conditions of mixed copper and zinc exposure, zinc, calcium, magnesium and potassium concentrations remain stable while sodium concentrations decrease. Also, there is new evidence that copper may act synergistically with metabolic analogues of calcium to disrupt calcium levels in the plasma of freshwater teleosts. Copper/sodium ratios in gill tissue are also presented as a possible biomarker for copper exposure.

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Chapter 4 - Post-mortem changes in gill metal concentrations in rainbow trout (Oncorhynchus mykiss) exposed to waterborne copper in fresh and brackish waters

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Abstract

Rainbow trout in fresh, 10 ppt and 20 ppt salinity water were exposed to sufficient waterborne Cu to induce moribundity in the animals (262, 346 and 589 μgL^{-1} Cu respectively). They were then euthanased and transferred to water of the same salinities with no added copper and left to depurate for 45 hours. Gill filaments were sampled at 0, 6, 18 and 45 hours and the metal concentrations of Cu, Zn, Ca, Mg and Na in the gills were assayed. Metal ratios were examined for their potential as biomarkers of exposure. At the time of transfer, the highest gill copper loads were found in the animals from the 10 ppt treatment (1.07 ± 0.2 μgg^{-1}, 1.41 ± 0.5 μgg^{-1}, 0.36 ± 0.1 μgg^{-1} for 0, 10 and 20 ppt treatments respectively, n = 6). Over time, all the metal concentrations in the gills equilibrated toward the environmental concentrations in all the treatments. This demonstrated gill metal ratios had limited application as biomarkers for copper exposure, post-mortem.

Additionally, animals from six fish kill episodes in Macquarie Harbour, Tasmania, were examined for copper and zinc concentrations in the gills and compared to animals from the laboratory trials. While metal ratios in the gills did not indicate copper toxicity as the cause of death, copper concentrations in the gills of Macquarie Harbour animals were higher than the concentrations that induced moribundity in animals in the laboratory trials (range; 1.27 ± 0.6 to 16.2 ± 9.6 μgg^{-1}, mean = 5.49 ± 5.6 μgg^{-1}, n = 6). This may be due to acclimation of the Macquarie Harbour animals to elevated waterborne copper. The levels of copper in the muscle tissue did not pose

an apparent health risks to consumers (range; 0.32 ± 0.3 to 1.59 ± 0.4 μgg^{-1}, mean = 0.84 ± 0.6 μgg^{-1}, n = 4).

Introduction

Copper, while being an essential element, is also toxic to aquatic and marine organisms. Copper may enter waterways through mining activity (Apte, et al. 1995), from prophylactic farm treatments (Carbonell and Tarazona, 1991), from industrial discharges (Lewis et al., 2002) or due to the action of acid rain lowering the pH of waterways and thereby solubilising minerals within the substrate (Sorensen, 1991). Many waterways around the world now have elevated copper levels, or are periodically exposed to pulses of copper at elevated levels (Eisler, 1997). Consequently there is considerable interest in developing a simple yet reliable biomarker for copper exposure, both for the monitoring of local water quality and for the investigation of fish kill episodes.

In freshwater systems, the ratio of copper to zinc, sodium or magnesium in the gill filaments of rainbow trout may demonstrate an animal has been exposed to elevated levels of environmental copper (Daglish and Nowak, 2002; Daglish et al., – *in press*). Cu/Mg and Cu/Na ratios were also able to indicate exposure to copper under conditions of mixed metal exposure (Daglish et al., in press). While ecotoxicological studies on the effects of copper on finfish to date have mainly investigated copper exposure in freshwater, elevated levels of metals, including copper, also occur in brackish, estuarine and marine environments (Eisler, 1997; Powell and Powell, 2001).

The effects and toxicity of metal exposure to teleosts are widely divergent between fresh, brackish and marine environments. Although there have been few experimental

studies of the toxicological effects of copper to finfish in brackish environments, it is recognised that higher salinities ameliorate the effects of copper poisoning (Taylor et al., 1996). The higher levels of cations in brackish and marine waters increase the competitive interactions between cations for binding sites at the gill surface (Playle, 1998), reducing the levels of copper bound to the gill surface. Animals are therefore able to withstand higher levels of dissolved copper in the water column. In an extensive series of tests of copper toxicity to fathead minnows *(Pimephales promelas)* in freshwater, combined data from Erickson et al., (1996) provided a mean 96-hr LC50 of 114.7 μgL^{-1} (range = 7.0 to 425.8 μgL^{-1}). Chakoumakos et al., (1979) tested the toxicity of copper to cutthroat trout *(Salmo clarki)* under various regimes of alkalinity and hardness and found the mean 96-hr LC50s ranged from 15.7 to 367 μgL^{-1} with a combined mean of 134.3 μgL^{-1}. As the water quality parameters of freshwater bodies may be highly variable due to chemical and physical differences between regions and seasons, these data have been used to demonstrate a full range of LC50s. In contrast, seawater may be considered a more homogenous chemical environment. Work by Taylor et al., (1985) recorded 96-hr LC50 for dab *(Limanda limanda)* in filtered seawater of 300 μgL^{-1} while the 96-hr LC50 for sea-mullet was 1400 μgL^{-1}. As metal ratios in the gills have indicated recent copper exposure in freshwater environments (Daglish and Nowak, 2002) (Daglish et al, *in press*), it would appear worthwhile to investigate their efficacy as an investigative tool for fish kills in higher saline environments.

Furthermore, there is a paucity of data relating laboratory studies of metal toxicology in aquatic environments to field situations and environmental conditions. Investigators of fish kills are frequently required to assess the cause of death of animals many hours or even days after mortality. Toxins in the water may be no longer detectable and elevated levels of metals bound to the gills of animals may leach into the water column. Additionally, with the cessation of homeostasis, the concentrations of other essential elements may be affected by diffusion processes across the gill epithelium. Copper concentrations of the gills are considered a potential biomarker for copper exposure (Taylor et al. 2000). However, if copper depurates from the gills prior to analysis, the effect of the exposure to copper will not be discernible by gill metal concentrations. It is therefore important to establish the effect on the concentrations of metals in the gills of animals killed by pollution events, to fish remaining in non-contaminated waters after death. Additionally, the rates of depuration and/or leaching of metals and metal residues from the gills will be affected by the water quality, including salinity, such that depuration and leaching rates will be affected differently in fresh, brackish and marine environments. It was therefore decided to correlate data from known, and potentially copper-related, fish kills in Macquarie Harbour, in south-western Tasmania, with data from the laboratory experiments.

Macquarie Harbour

Macquarie Harbour, located on the western coast of Tasmania, is approximately 32 km long and 8 km wide with an area of 276 km^2 and a mean depth of approximately

20 m. There is a narrow opening to the Southern Ocean at the north-western corner of the harbour. Fresh water flows into the harbour from the King River in the north-eastern area of the harbour and the Gordon-Franklin River system in the south-eastern section. Tidal movements of the harbour are generally less than 1m. The water of Macquarie Harbour is distinctly stratified into two layers. The surface waters of the harbour, defined as 0 - 5 m, are characterised by highly variable salinities and high levels of organic carbons in the form of tannic and humic substances giving the surface waters a distinctive brown "tea-like" appearance. The remainder of the water column is saline. The variability of the surface water's salinity and organic content is mainly due to rainfall, changes in the flow of freshwater from the two river systems and mixing by wind (Carpenter et al., 1991; Koehnken, 1996).

Macquarie Harbour has been heavily polluted by discharge from the Mt Lyell copper mine in South-western Tasmania since the commencement of mining operations in 1883. The Mt Lyell copper mine is located on the Queen River approximately 15 km above the confluence of the King and Queen Rivers and a further 25 km to Macquarie Harbour. It has been estimated that the Queen River has received approximately 100 million tonnes of mine tailings since the commencement of mining (Carpenter et al., 1991) and a distinct alluvial 'fan' of tailings is visible at the confluence of the of the King River and Macquarie Harbour. Remediation works at the mine site, including the King River and Macquarie Harbour have been in progress since 1993.

The aims of this experiment were to examine: the depuration rate of Cu from the gills of rainbow trout, post-mortality, after exposure to lethal levels of copper in fresh and brackish waters; the concentrations of Zn, Ca, Mg and Na in the gills of rainbow trout that have remained in a non-contaminated body of water post-mortality induced by exposure to lethal levels of copper in fresh and brackish waters; the efficacy of metal ratios as biomarkers when animals have remained in a non-contaminated body of water post-mortality and subsequent to exposure to lethal levels of copper in fresh and brackish waters; and data from fish kills in a brackish environment in comparison to experimental laboratory data to evaluate the use of copper/metal ratios under field conditions.

Methods and materials

Eighteen rainbow trout (275.7 ± 41.3 g) were obtained from the Aquaculture Centre, School of Aquaculture, University of Tasmania. They were randomly separated into 3 groups of 6 fish and acclimated in either moderately hard freshwater, 10 ppt brackish water or 20 ppt brackish water for eight days. Acclimation was conducted in 300 L static, aerated tanks. Complete water changes were conducted every second day. Salinity was measured using a salinity refractometer. Concentrations for Na, Ca, and Mg were supplied by Mr. S. Roberts of the School of Aquaculture, University of Tasmania for the freshwater treatment or calculated from available literature (Bidwell and Spotte, 1985) for the 10 ppt and 20 ppt salinity treatments. Water quality of the acclimation tanks, monitored daily, were as follows: freshwater; salinity = 0 ppt, [Na] = 26.1 mgL^{-1}, [Ca] = 19.9 mgL^{-1}, [Mg] = 47.9 mgL^{-1}, pH = 7.9 ± 0.1, T = 18.4 ± 1.0 °C, D.O. = 8.0 ± .04 mgL^{-1}, NO_2 < 0.1 mgL^{-1}, NO_3 = 0, total hardness = 107.0 ± 1.8 mgL^{-1} as $CaCO_3$, Cu < 5.0 μgL^{-1}: 10 ppt brackish water; salinity =10.4 ± 0.5, [Na] = 3003 mgL^{-1}, [Ca] = 114.4 mgL^{-1}, [Mg] = 318.1 mgL^{-1}, ppt, pH = 7.9 ± 0.1, T = 18.5 ± 0.9 °C, D.O. = 8.0 ± 0.4 mgL^{-1}, NO_2 < 0.1 mgL^{-1}, NO_3 = 0, Cu < 5.0 μgL^{-1}: 20 ppt brackish water; salinity = 20.3 ± 0.5, [Na] = 5995.5 mgL^{-1}, [Ca] = 228.4 mgL^{-1}, [Mg] = 770.8 mgL^{-1}, ppt, pH = 7.9 ± 0.1, T = 18.4 ± 0.9 °C, D.O. = 7.9 ± 0.5 mgL^{-1}, NO_2 < 0.1 mgL^{-1}, NO_3 = 0, Cu < 5.0 μgL^{-1}.

Experimental Design

A repeated measures design was used with time and salinity as independent variables and gill concentrations of the metals Cu, Zn, Ca, Mg and Na as the dependent

variables. After acclimation the tanks were replenished with approximately 300 L of water of the appropriate salinity. The fish were exposed to toxic levels of copper by the addition of 353 mg, 530 mg and 708 mg of $CuSO_4.5H_2O$ to the fresh, 10 ppt salinity and the 20 ppt salinity treatments respectively. Measured copper levels were 262.0 ± 22.5 μgL^{-1} (0 ppt, n = 3), 346.1 ± 17.3 μgL^{-1} (10 ppt, n = 3) and 589.2 ± 7.6 μgL^{-1} (20 ppt, n = 3). Animals were exposed for 48 hours by which time they were moribund, as indicated by loss of balance while swimming. They were then sacrificed by exposure to 10 mgL^{-1} of benzocaine. After sacrifice the first gill arch was excised from each animal and placed on ice prior to being stored at – 20 °C. The carcasses were returned to 300 L tanks of uncontaminated water of the same water quality as the acclimation tanks. Successive gill arches were sampled after 6, 18 and 45 hours by which time the animals were showing early stages of decomposition. The gills filaments were white, the carcasses were displaying a general loss of colour and the flesh was soft to touch.

Field Data

Field data were supplied by the Fish Health Unit, Animal Health Laboratory of the Department of the Industry, Water and the Environment (DPIWE), Tasmania. Data were from fish kill episodes in Macquarie Harbour, Tasmania, between 1991 and 1995. Cases were only considered where copper and zinc data for gill tissue was provided. Copper analysis of liver and muscle tissue were also supplied. To assure the statistical rigour of the procedure, data were used only if more than 5 or more mortalities were recorded from a single episode. Water quality data for the

corresponding time periods from Macquarie Harbour were supplied by the Toxicology Unit, DPIWE, Tasmania. Metal analyses of tissue and water samples were performed by flame and graphite furnace AAS, respectively, using standard laboratory procedures.

Metals Analysis

Gill filaments were dissected from the gill arch and digested in 5 mL of concentrated HNO_3 in Teflon digest vessels in a CEM MDS-2000 microwave digester. Between 50 and 200 mg of filamentous tissue was used for each digest. Digest vessels and glassware were washed in 10% HNO_3 and rinsed in deionised water prior to use.

Metal analysis was performed using a Varian Spectra AA 300 Atomic Absorption Spectrometer (AAS): calcium analysis was performed using a nitrous oxide/acetylene flame; copper, zinc and magnesium samples were analysed using an air/acetylene flame; sodium analysis was performed by emission spectrometry with an air/acetylene flame. Standards were run prior to and after each set of analyses to prevent errors due to drift while analysing samples. Samples of gill tissue were spiked with 1 mgL^{-1} of Cu^{2+} and recovery rates were determined. These ranged from 103% to 109%. National Institute of Standards and Technology bovine liver samples (NIST 1577b) were run to verify the laboratory methods. All data presented are for wet weight of tissue and given on a wet weight (w/w) basis.

Statistical Analysis

Results were analysed by the software package SPSS v10.0. Differences between treatments were considered significant at the level of $\alpha = 0.05$, using Pillai's Trace. All data were tested for equality of error variance using Levene's Test. Normality of data were confirmed using pp plots and histograms of the variables.

Analysis was conducted within each salinity regime by univariate ANOVA, with time as the independent variable and the individual metal levels as the dependent variables. All data were transformed using natural logarithms to satisfy the assumption of homogeneity of variance between groups. *Post hoc* analyses were performed using Scheffe's method.

Comparisons of depuration rates and metal levels between the different salinities were conducted by using a repeated measures profile analysis where the gill arches were considered as the repeated units of measurement over time. A two-factor mixed general linear model was used where salinity was the between-subjects variable and time was the withi- subjects variable for the various metal levels (Ho 2000). To enable comparisons between salinities, all data were normalised within each animal by dividing the metal level of each arch by the metal level of the first arch thereby making the first sample (time 0) equal to one and each subsequent sample a proportion of the initial value (times 2, 3 & 4 each respectively 6, 18 and 45 hours post-mortality and transferral to clean water of the appropriate salinity).

The ratios of the concentrations of zinc, calcium, magnesium or sodium to copper concentrations in the gills of the animals were calculated. Comparisons between animals in the different salinities were made as well as comparisons between animals from Macquarie Harbour and experimental animals. All metal ratios were determined using the formula:

$(Cu\ (\mu g.g^{-1})/M\ (\mu g.g^{-1}))*1000,$

where M represents the metal of interest and a multiplier of 1000 is used to increase the magnitudes of the ratios to simplify further analysis. Post hoc tests were conducted using Tukey's test.

Results

Laboratory Data

Gill metal levels

Trial A: Freshwater

The concentrations of copper and sodium measured in the gills decreased significantly over time in the freshwater treatment while the magnesium concentration of the gills significantly increased. No significant change was recorded in the levels of zinc. A significant result was noted for calcium at the alpha = 0.05 level, however *post hoc* tests did not differentiate between the mean values at the different times and the result was treated as non-significant. (Table 1; Figs. 1 – 5).

The levels of copper in the gills decreased rapidly and significantly from the initial value of 1.07 ± 0.2 $\mu g.g^{-1}$ of tissue at 0 hours to 0.45 ± 0.1 $\mu g.g^{-1}$ of tissue at 6 hours, after which little further change was recorded. The mean values recorded for zinc showed little difference between 0 and 6 hours, however increased from 20.2 ± 9 $\mu g.g^{-1}$ of tissue at 6 hours to 30.2 ± 17 $\mu g.g^{-1}$ of tissue at time 18 hours, before declining to 21.3 ± 7 $\mu g.g^{-1}$ of tissue at 45 hours post-mortality. The mean recorded values for calcium in the gills of the fish held in freshwater increased steadily during the post-mortality period from 356.4 ± 107 $\mu g.g^{-1}$ of tissue at 0 hours to 670.7 ± 277 $\mu g.g^{-1}$ of tissue 45 hours after mortality. Magnesium levels followed a similar pattern to calcium, increasing steadily over time from the 41.0 ± 6 $\mu g.g^{-1}$ at 0 hours to 74.4 ± 29 $\mu g.g^{-1}$ of tissue 45 hours post-mortality. The initial and final readings were significantly different from each other. Sodium levels decreased rapidly and

Table 4.1 Statistical analysis of the effects of time on the levels of Cu, Zn, Ca, Mg and Na in the gill filaments of rainbow trout. Animals were exposed to elevated copper levels in freshwater until moribund and then left in clean water post-mortality for 45 hours. Gill arches were removed for metal analysis at times 0, 6, 18 & 45 hours.

Metal	**n**	**F value**	**p-value**
Cu	6	13.217	< 0.001
Zn	6	0.968	0.427
Ca	6	3.106	0.05
Mg	6	3.787	0.027
Na	6	35.048	<0.001

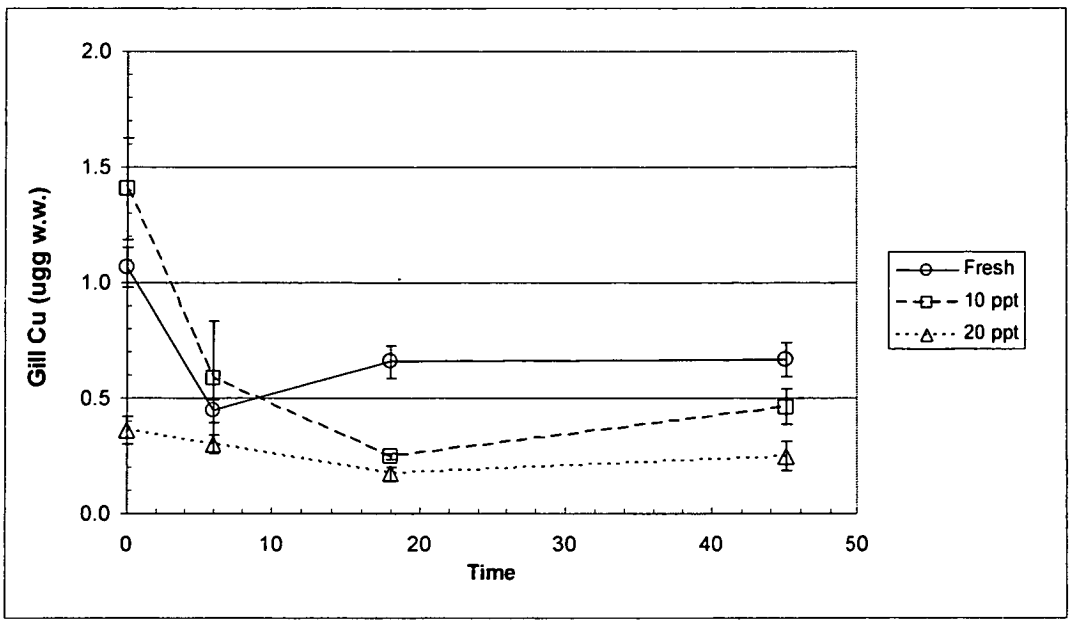

Fig 4.1 Mean copper concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

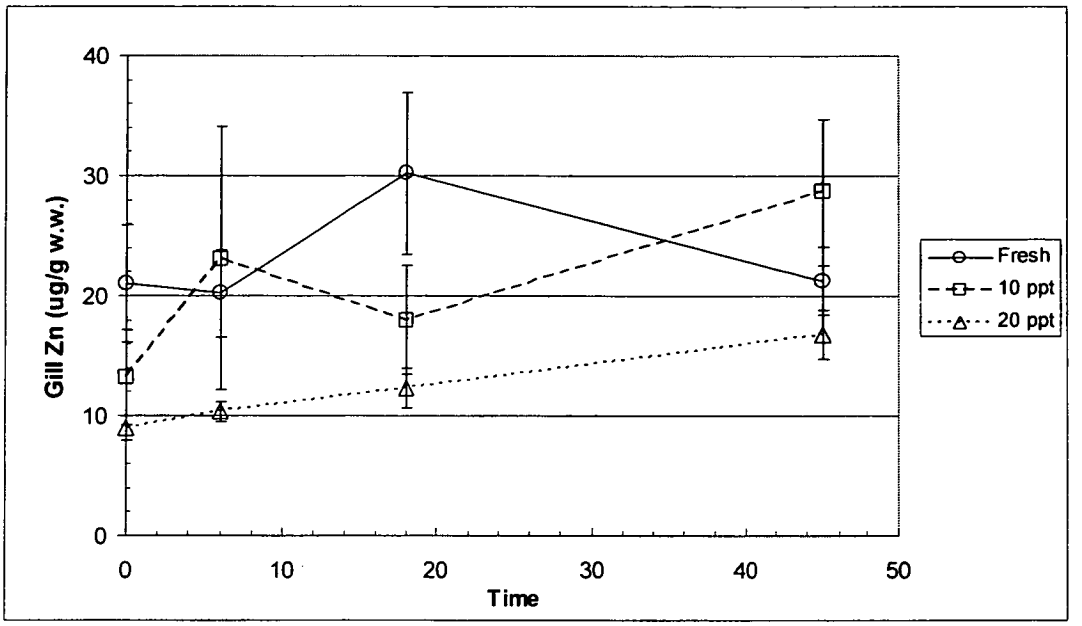

Fig 4.2 Mean zinc concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

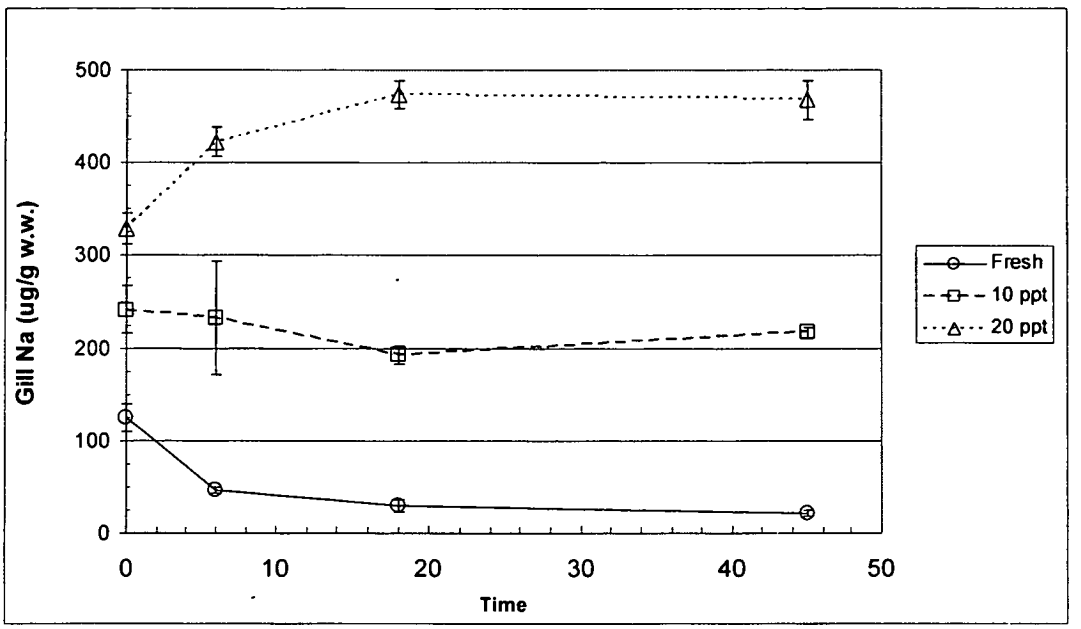

Fig 4.3 Mean sodium concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

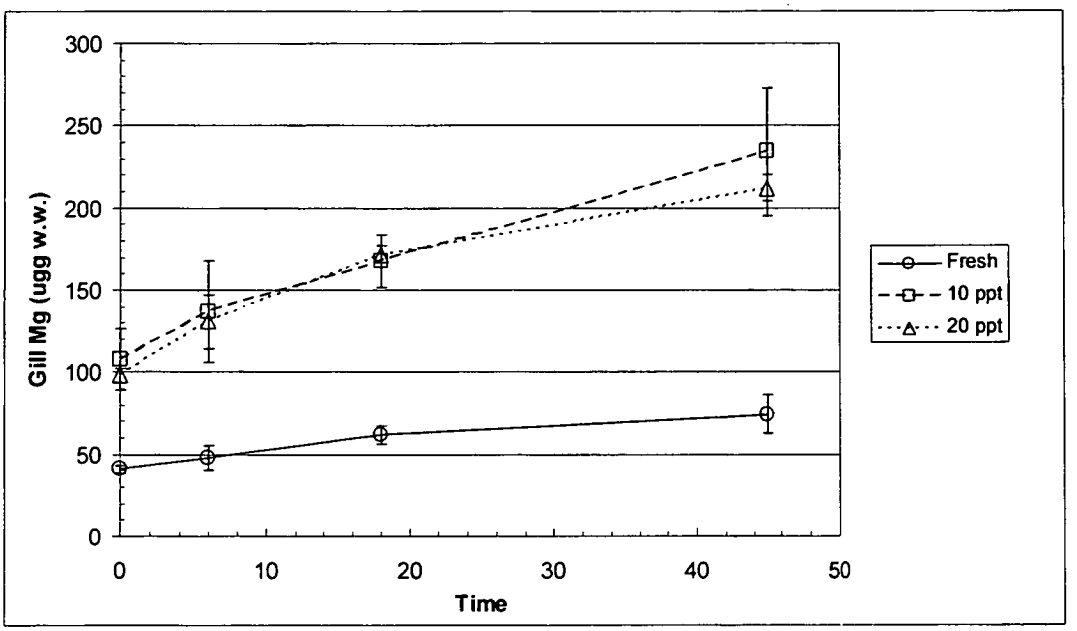

Fig 4.4 Mean magnesium concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

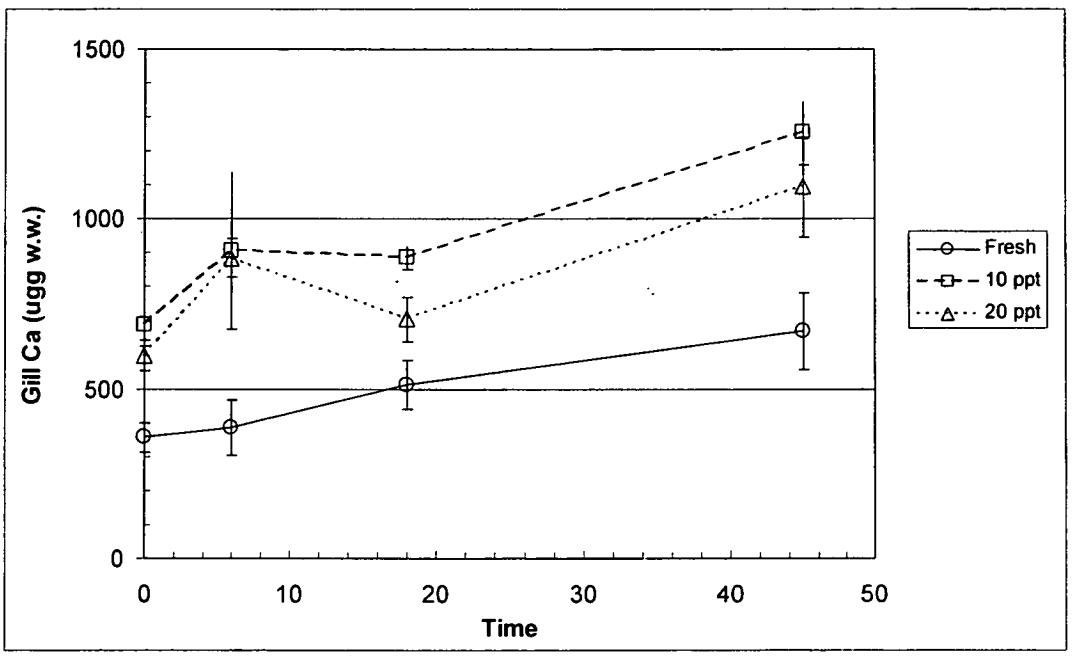

Fig 4.5. Mean calcium concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

significantly from their peak at time 0 of 124.6 ± 36 $\mu g.g^{-1}$ of tissue to 46.2 ± 8 $\mu g.g^{-1}$ of tissue at 6 hours. There was a further slight decrease by the end of the trial at time 45 hours post-mortality to 14.7 ± 5 $\mu g.g^{-1}$ of tissue.

Trial B: Brackish water (10 ppt salinity)

In the 10 ppt brackish water trial a significant decrease was recorded over time for the levels of copper, while calcium and magnesium in the gills of rainbow trout post-mortality increased significantly. The concentrations of gill zinc or sodium were not significantly altered (Table 2; Figs 1 -5).

The mean recorded levels of copper decreased from 1.41 ± 0.5 $\mu g.g^{-1}$ of tissue to a minimum value of 0.25 ± 0.04 $\mu g.g^{-1}$ of tissue after 18 hours in clean 10 ppt water. A slight recovery to 0.46 ± 0.2 $\mu g.g^{-1}$ of tissue occurred by 45 hours. Mean zinc scores in the gills of the animals increased over the course of the depuration period from 13.2 ± 10 $\mu g.g^{-1}$ of tissue to 28.7 ± 15 $\mu g.g^{-1}$ at time 45 hours post-mortality. Calcium levels in the gills of rainbow trout increased steadily from 689.4 ± 157 $\mu g.g^{-1}$ of tissue to 1253.6 ± 227 $\mu g.g^{-1}$ of gill filament tissue 45 hours post-mortality. The initial and final calcium levels were significantly different from each other. Magnesium levels in the gills showed a similar response to calcium, increasing steadily from 107.9 ± 47 $\mu g.g^{-1}$ of tissue to 234.0 ± 95 $\mu g.g^{-1}$ by 45 hours post-mortality, and the initial and final magnesium levels were significantly different. Sodium mean values decreased slightly, but not significantly, over the period of depuration from 241.2 ± 63 $\mu g.g^{-1}$ of tissue to 216.9 ± 14 $\mu g.g^{-1}$ of gill tissue.

Table 4.2 Statistical analysis of the effects of time on the levels of Cu, Zn, Na, Mg and Ca in the gill filaments of rainbow trout exposed to elevated copper levels in 10 ppt brackish water until moribund and then left in clean water post-mortality for 45 hours. Gill arches were removed for metal analysis at times 0, 6, 18 & 45 hours.

Metal	**n**	**F value**	**p-value**
Cu	5	1.234	0.323
Zn	5	0.908	0.455
Ca	5	3.299	0.041
Mg	5	3.798	0.026
Na	5	0.382	0.767

Trial C: Brackish water (20 ppt salinity)

The levels of all metals other than copper measured in the gill filaments of rainbow trout exposed to acute copper levels in the 20 ppt brackish water trial increased significantly over time after being transferred to clean water post-mortality (Table 3; Figs 1 – 5). No significant change was recorded for the mean gill copper concentrations.

Copper mean values at time 0 were 0.365 ± 0.14 $\mu g.g^{-1}$ of gill filament, and decreased slightly to 0.250 ± 0.15 $\mu g.g^{-1}$ 45 hours after mortality and transferral to clean water. Zinc levels increased steadily from an initial value 9.00 ± 2.6 $\mu g.g^{-1}$ of tissue to a final value of 16.8 ± 5 $\mu g.g^{-1}$. Zinc levels at times 0 and 6 hours were significantly different to the final recorded value. Calcium levels increased from their lowest result at time 0 of 597.6 ± 112 $\mu g.g^{-1}$ to 885.6 ± 143 $\mu g.g^{-1}$ 6 hours later. They then decreased to 706.0 ± 160 $\mu g.g^{-1}$ 18 hours post-mortality and then increased again to 1094.6 ± 366 $\mu g.g^{-1}$ when the experiment was terminated 45 hours after mortality and transferral. The levels of calcium in the gill filament tissue at times 0 and 18 hours were significantly lower than the final value. Magnesium levels also increased steadily and significantly, from 98.0 ± 10 $\mu g.g^{-1}$ at time 0, to 131.1 ± 40 $\mu g.g^{-1}$ at time 6 hours, 171.8 ± 12 $\mu g.g^{-1}$ at time 18 hours and a final value at time 45 hours of 212.3 ± 19 $\mu g.g^{-1}$. Magnesium levels in the gill filaments at time 0 were significantly lower than at time 18 or 45 hours and the result for magnesium at time 6 hours was also significantly lower than at 45 hours post-mortality. Sodium levels in the gills of the animals in this treatment increased rapidly after death from the original level at time 0

of 329.6 ± 40 $\mu g.g^{-1}$ to 422.5 ± 39 $\mu g.g^{-1}$ 6 hours after mortality. The mean recorded values of sodium increased slightly but not significantly thereafter to a final level of

Table 4.3 Statistical analysis of the effects of time on the levels of Cu, Zn, Na, Mg and Ca in the gill filaments of rainbow trout exposed to elevated copper levels in 20ppt brackish water until moribund and then left in clean water post-mortality for 45 hours. Gill arches were removed for metal analysis at times 0, 6, 18 & 45 hours.

Metal	**n**	**F value**	**p-value**
Cu	5	2.868	0.062
Zn	5	5.333	0.007
Ca	5	5.915	0.005
Mg	5	26.307	<0.001
Na	5	14.934	<0.001

468.0 ± 51 $\mu g.g^{-1}$ of gill filament tissue. The initial value at time 0 was significantly lower than all the other sodium scores.

Profile Analysis

A significant difference was observed between the different salinities for the depuration rates of copper (Pillai's Trace, 0.827; F = 3.288; p = 0.014). The 10 and 20 ppt brackish groups were autonomous of each other while the freshwater treatment was not significantly different to either group (Fig 6).

The rates of depuration for sodium also were significantly different between salinity treatments (Pillai's Trace, 1.364; F = 10.002; p < 0.001). All three treatments were independent of each other (Fig 7).

No significant differences of parallelism were recorded for any of the other metals measured in the gill filaments of the treated rainbow trout.

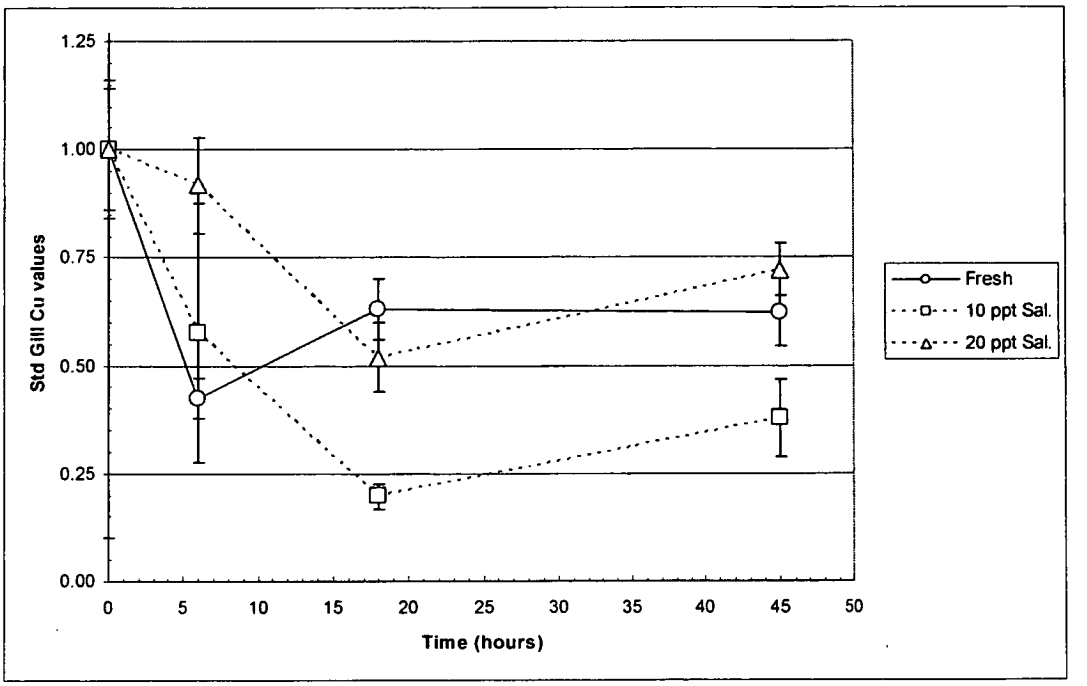

Fig 4.6. Standardised mean copper concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

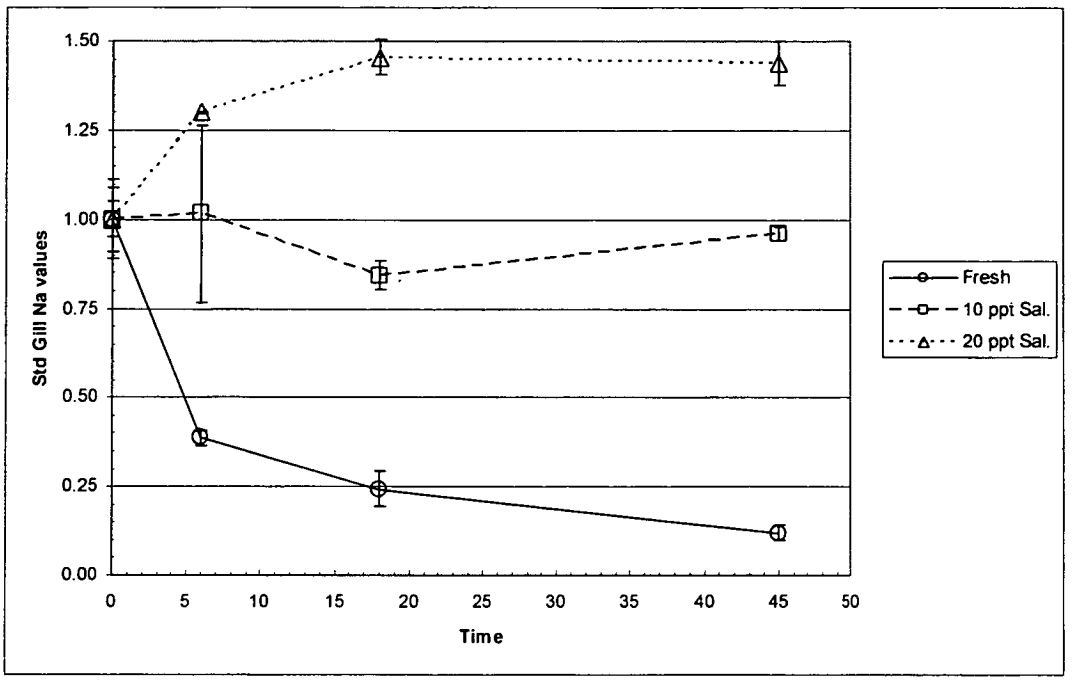

Fig 4.7 Standardised mean sodium concentrations, with standard errors, in the gill filaments of rainbow trout exposed to acute waterborne copper until moribund in fresh, 10 ppt and 20 ppt brackish waters then left in non-contaminated water for 45 hours post-mortem

Gill Metal Ratios

Results for time 0 hours are presented in table 4.

Trial A: Freshwater

In the 0 ppt treatment the Cu/Zn ratios decreased significantly ($F = 3.771$, $p = 0.027$) from the highest value at time 0 of 66.42 ± 39.6 to 27.26 ± 18.4 at time 6 hours. The average mean values of the Cu/Zn ratios increased slightly thereafter to a final value of 33.53 ± 11.0 at 45 hours *post-mortem*, which was no longer significantly different to the original value at time 0. The Cu/Na ratios of the freshwater carcasses showed no significant change from the initial value of 9.14 ± 2.8 up to time 6 hour. However, a significant increase occurred between time 6 and 18 hours as the ratio increased to 25.10 ± 12.8 and then again from time 18 to 45 hours as the ratio increased to 46.86 ± 17.9 ($F = 21.12$, $p < 0.001$).

The highest value for the Cu/Mg ratios of 27.00 ± 8.3 was recorded at time 0 after which a rapid and significant decrease in the ratios occurred to 10.9 ± 5.9 at time 6 hours post-mortality ($F = 10.86$, $p < 0.001$). The mean values of the ratios did not alter significantly thereafter. The Cu/Ca ratios also recorded the highest value of 3.47 ± 1.9 at time 0 and then declined rapidly and significantly to 1.48 ± 0.9 after which no further significant changes were recorded for the mean ratios ($F = 4.97$, $p = 0.010$).

Table 4.4 Gill Cu/M ratios in the gills of rainbow trout moribund from waterborne copper exposure in waters of 3 different salinities. Mean values are presented with standard deviations in parentheses.

Gill Cu/M ratio	Fresh (0 ppt)	10 ppt	20 ppt
Cu/Zn	66.4 (39.6)	192.81 (174.72)	41.43 (14.74)
Cu/Ca	3.47 (1.93)	6.38 (2.71)	0.61 (0.22)
Cu/Mg	27.00 (8.34)	15.87 (5.82)	5.40(1..31)
Cu/Na	9.14 (2.84)	2.23 (1.07)	1.13 (0.46)

Trial B: Brackish water (10 ppt salinity)

Significant differences were seen over time for the Cu/Zn and Cu/Mg ratios in the 10 ppt treatment but not for the Cu/Na or Cu/Ca ratios.

The greatest value for Cu/Zn ratios of 192.81 ± 174.7 was recorded at time 0 hours after which mean values steadily declined to the minimum recorded value of 16.73 ± 7.1 at time 45 hours *post-mortem.* The final Cu/Zn value was significantly different to the initial reading ($F = 3.631$, $p = 0.036$) although no other times were significantly affected according to *post-hoc* analysis.

The Cu/Mg ratios also showed significant differences over time ($F = 4.475$, $p = 0.018$). The highest reading of 15.87 ± 5.8 was recorded at time 0. This value declined to 8.14 ± 12 .3 by time 6 hours and then further decreased to 1.64 ± 0.4 at time 18 hours after which no further differences in mean values were recorded. *Post hoc* tests grouped the two final readings separately from the initial reading.

Trial C: Brackish water (20 ppt salinity)

The Cu/Zn ratios in the 20 ppt treatment declined significantly over time ($F = 6.648$, $p = 0.003$). The highest value of 41.43 ± 14.7, recorded at time 0, declined to 29.51 ± 8.4 at time 6 hours and further declined to 16.65 ± 12.1 at 18 hours post mortality. The ratio at time 18 hours was significantly less than at time 0 hours. No further changes in the mean values were seen after 18 hours. Cu/Na ratios also decreased

significantly over time (F = 6.461, p = 0.003) from their highest value of 1.13 ± 0.5 at time 0 to the lowest value recorded at time 18 hours of 0.36 ± 0.1 after which the mean value of the ratios increased slightly to 0.55 ± 0.3 45 hours *post-mortem. Post hoc* tests grouped the final two readings separately to the initial reading. A similar pattern described the Cu/Mg ratios, decreasing from their highest value of 3.69 ± 1.3 at time 0 to their lowest value of 1.02 ± 0.5 before showing a slight increase at time 45 hours to 1.20 ± 0.7. The differences over time were significant (F = 4.824, p = 0.011) and post hoc tests grouped the final two readings at time 18 and 45 hours separately from the initial reading. This pattern was again repeated for the Cu/Ca ratios where the highest value, recorded at time 0, was 0.61 ± 0.2 and the lowest value of 0.26 ± 0.16 was recorded at time 18 hours. The differences over time were significant (F = 4.939. p = 0.10) and the readings at time 18 and 45 hours post-mortality were grouped separately to the value at time 0 after *post-hoc* analysis.

Field Data

Fish Kill Episodes

Between 19 June, 1995 and 4 January, 2001, 6 fish kill episodes occurred in Macquarie Harbour where 5 or more animals died from unknown causes and sufficient data were recorded for inclusion in this report (Table 5). Due to the history of the Mt Lyell Copper mining operations, copper poisoning was suggested as a potential cause of mortality.

Table 4.5 Cu and Zn levels in the gills, liver and muscle of salmonids from fish kill episodes in Macquarie Harbour, 1995 – 2001, plus gill Cu/Zn ratios. All data are presented as means values with standard deviations in parentheses.

Case	n	Gill Cu ($\mu g.g^{-1}$ w.w.)	Gill Zn ($\mu g.g^{-1}$ w.w.)	Gill Cu/Zn ratio	Liver Cu ($\mu g.g^{-1}$ w.w.)	Muscle Cu ($\mu g.g^{-1}$ w.w.)
953322	18	16.2	na	na	325.5	na
		(9.6)			(97.0)	
960843	10	5.97	na	na	1669.5	na
		(1.0)			(401.0)	
012421	10	5.47	1146.8	5.75	542.7	1.59
		(1.0)	(517.8)	(2.8)	(134.5)	(0.45)
012800	10	2.54	755.1	4.27	753.1	1.08
		(0.52)	(448.2)	(2.2)	(159.3)	(0.43)
012802	5	1.27	340.0	4.45	1047.3	0.38
		(0.64)	(185.8)	(3.1)	(185.6)	(0.35)
012803	10	1.46	668.2	4.92	830.0	0.32
		(0.80)	(549.0)	(8.8)	(263.0)	(0.33)

Tissue metal levels and gill Cu/Zn ratios

Mean gill copper levels for the various fish kill episodes ranged from 1.27 ± 0.64 µg.g-1 to 16.2 ± 9.6 µg.g-1 while mean gill zinc levels ranged from 340.0 ± 185.8 µg.g-1 to 1146.8 ± 517.8 µg.g-1. Cu/Zn ratios in the gills ranged from 4.27 ± 2.2 to 5.75 ± 2.8. Hepatic copper levels ranged from 325.5 ± 97.0 µg.g-1 to 1669.5 ± 401.0 µg.g-1 while copper levels in muscle tissue ranged from 0.32 ± 0.3 µg.g-1 to 1.59 ± 0.45 µg.g-1.

Discussion

Laboratory Trials

Moribundity was chosen as the preliminary end point of these trials and the point at which to transfer the animals to clean waters for the depuration period for two reasons. The first of these was to be sure the animals were acutely affected by the toxic conditions of their environment thereby mimicking a fish kill scenario and secondly as it enabled analysis of the relevance of the results as bio-markers for copper induced mortality. Although different levels of additional copper were required in each treatment to induce moribundity within similar time periods, our experimental method is in accord with the principles of the Biotic Ligand Model (B.L.M.) (Di Toro et al., 2001; Santore et al., 2001). The BLM has emerged as a powerful and probable mechanism for explaining metal toxicity. It predicts occurrence of mortality when a metal-biotic ligand complex at the gill's surface reaches a critical concentration, disrupting regulation of the blood ions at the gill epithelium. Mortality is thereby related to the level of metal bound at the gill surface, rather than the level of metal in the water column (Meyer et al., 1999). The amount of copper bound by the gills is considered primarily as a function of competitive interactions between the toxic metal species and other cations in the water column, and the available binding sites at the gill surface. In waters of higher ionic composition, the greater level of competitive interactions should provide greater protection against the effects of the toxic metal species (Pagenkopf, 1983).

As the freshwater treatment had the lowest ionic composition we expected the freshwater animals to record the highest levels of gill copper at time 0 hours and the lowest amount of copper to be bound by the 20 ppt treatment. Our prediction was supported by (Taylor et al., 2000) who found the maximum binding capacity of the gills of rainbow trout (B_{max}) decreased as water hardness increased. However our data do not support our prediction as the highest level of gill copper was recorded in the 10 ppt treatment.

Wilson and Taylor (1993) found rainbow trout that were acclimated to 33% seawater (~12 ppt salinity) and exposed to 6.3 $\mu mol.L^{-1}$ (400 μgL^{-1}) Cu suffered from impairment of their branchial ionoregulatory function. This was indicated by a significant increase in plasma Na and Cl. However, no effect was observed in animals acclimated to full strength seawater. The authors suggested the results of the 33% trial were due to gill permeability changes after displacement of surface bound Ca, whereas the animals in full strength seawater were buffered from any deleterious changes to the permeability of the gills by the higher concentrations of calcium present in the full strength seawater. Taylor et al., (2000) also suggested a role for calcium in regulating gill membrane permeability and the stability of membrane proteins which may lead to alterations in the binding affinity and the $B_{(max)}$ of the gills.

Our data also found a greater toxic effect, as indicated by the levels of copper bound to the gills, in animals at levels of salinity close to isotonic to the animal's internal

environment, i.e. approximately 33% of seawater (Wilson and Taylor, 1993). However, if the calcium displacement argument is the main causative factor for the differences in the binding of gill copper, then the highest levels of copper would be found in the freshwater treatment. We believe physiological factors may be more important in these results. While freshwater animals require the active uptake of Na from the environment, marine animals must extrude Na across the gill epithelium (Evans, 1993; Karnaky Jr, 1998). Little is known about branchial iono-regulation in animals in an isotonic environment.

At time t = 0, assuming there is negligible active transport of Na under isotonic conditions, the gill membrane may be modelled as the ion exchange sites of an ion exchange resin. An alternative explanation then may be posited. If sodium is being actively transported across a membrane (when the surrounding water is either hypo- or hypertonic to the fish) it will elute copper from an ion-exchange site, and the copper will move in the direction of the Na ion movement. However in a system in equilibrium, i.e. where the level of Na ions is equal on each side of the membrane, there will be minimal Na movement. Under these conditions, Cu remains on the membrane and reaches the maximum concentration on the membrane. Such a mechanism where acute Na ion movement through a membrane elutes Cu ions from an exchange site would explain the maximum concentration of Cu on the gill membrane in the 10 ppt salinity treatment in these experiments.

The authors therefore believe the mechanistic approach of BLM may not account for the physiological changes in ion regulation at the gills when animals are acclimated to brackish or saline waters. Furthermore, the current model of copper toxicology in freshwater, being based primarily upon the disruptive effects of Cu on branchial sodium regulation is not suitable for marine and brackish acclimated animals where the mechanism of ion-regulation is opposite to that of freshwater animals. It is also noted that although different levels of copper were added to the various treatments to induce moribundity within the same time period, the difference in the ionic strength of the treatments due to the additional copper is neglible when compared to the changes in ionic strength due to the various salinities.

From time 0, the copper levels in all treatments declined rapidly as the gill tissue equilibrated with the environment. The rate of depuration was most rapid in the freshwater treatment, having reached its minimum value in 6 hours while the two brackish treatments were slower in depurating and did not appear to approach equilibrium until 18 hours *post-mortem*.

For the metals zinc, calcium and magnesium, the gill levels increased steadily during the depuration period in the 10 and 20 ppt brackish water treatments. This can be explained simply by equilibrium reactions at the gill surface with the aqueous environment. All three of these metals exist in brackish waters at considerably higher levels than the intra-cellular requirements of fish homeostasis. Once the animals are

dead and the epithelial integrity is lost, there will be a movement to equilibria of all the cellular components.

Sodium provides a particularly succinct example of this effect (Fig 5). While all three groups of animals exhibit the normal range of sodium in the gills for fresh or brackish water fish, once the animal is deceased the sodium levels quickly stabilise at a level assumed to be in equilibria with their environment. Sodium levels are stable in all three groups within 18 hours of the death, whereas for calcium and magnesium, it appeared the gill tissue was still moving toward a final equilibrium value 45 hours *post-mortem*.

It is noteworthy that the levels of the metals in the gill tissue did not equilibrate to the exact values of their environment, i.e. to the concentration of the surrounding waters. However, this may be explained by the association of the metals with the tissue of the gills. While the homeostatic processes that maintain the animal's ion concentrations are no longer active *post-mortem*, the physical properties of the proteins in binding the metals are still effective. Therefore the final equilibrated concentration of the metals associated with the tissue will be dependent of the binding affinity of the metal with the tissue and the concentration of the metal in the surrounding environment. Although the concentration of the metals in the tissue move towards the concentration of metal in the environment, they do not equal the concentrations of the metal in the environment.

Metal Ratios

Previous work has demonstrated the potential of metal ratios in the gill tissue to act as biomarkers for exposure to acute levels of copper in freshwater fish (Carbonell and Tarazona, 1993; Daglish and Nowak, 2002) and also under conditions of mixed copper and zinc exposure (Daglish et al., *in press*). Copper ratios to the metals zinc, sodium and magnesium provided the most significant descriptors of copper exposure in freshwater. Data from this research support the use of gill metal ratios as a means of determining if copper toxicity is responsible for a fish kill in the period of time immediately post-mortem. However, it is important to note that the final ratio of gill metal levels in a deceased animal will not be the same as the basal level recorded in a live animal after exposure to copper. The levels of metals in the carcass will reflect an environmental equilibrium whereas the basal level in a live animal will reflect the homeostatic requirements of the animal.

The Cu/Zn ratios of the freshwater fish at time 0 were significantly higher than in unexposed (control) fish from earlier studies in this laboratory and comparable to the levels of animals exposed to elevated ambient copper. (Daglish and Nowak 2002; Daglish et al. *in press)*. Copper residues declined rapidly post-mortem whereas zinc levels did not alter significantly across the same period. Correspondingly the Cu/Zn ratios of the gills declined rapidly and significantly between time 0 and 6 hours after which no further decrease was recorded. The levels after 6 hour did not differ significantly from control groups of our earlier experiments (Daglish and Nowak 2002; Daglish et al. *in press)*. The Cu/Zn ratio may therefore be a useful indicator of

copper toxicity at the time of death and shortly thereafter, but within 6 hours the ratios would appear to return to levels seen in unexposed animals. As a biomarker this ratio is therefore useful in moribund animals that have moved away from a contaminated water body, or have recently died, but will have limited applicability after death.

In the 10 ppt and 20 ppt trials the rate of depuration is less rapid and the Cu/Zn ratios do not plateau until 18 hours after death, providing a larger time frame in which the ratios may be used for identifying *a prior* copper exposure. In both these treatments the zinc levels of the gill tissue increased over time in the carcasses as equilibrium was established between the tissue and environment which would contribute to the slower change in the ratios. There may also be a 'preservative effect' due to the higher saline environment (Munday and Jaisankar 1998). The ratios appear to have plateaued by 45 hours in both treatments.

The Cu/Na ratios reflect clearly the effect of the environment on the tissue metal levels. In the freshwater treatment, the ratios are stable through the first 6-hour period and then increase four to five-fold over the next 39 hours as the sodium within the gill tissues moves into the surrounding waters. While the changes are significant, the utility of this ratio as a biomarker is limited by the magnitude of the change in sodium levels, which may be sufficient to mask the effects of the altered levels of copper in the gills. In context of our ion exchange theory, it is necessary to consider why Cu is

not also 'eluted' as the sodium moves into the environment. Two plausible explanations may be considered. Firstly, osmosis, principally involving bulk sodium movement, would dominate the equilibration of the tissue with the surrounding environment with the displacement of copper occurring subsequent to this ionic movement. Secondly, the Cu and Na ions will display different binding affinities to the gill tissue. Copper binding to proteins may be characterised by its ability to chelate to multi-attachment sites and will therefore be less easily detached from the tissues after death than the mono-valent sodium.

The opposite effect upon Cu/Na ratios was seen in the 20 ppt treatment to that in the 0 salinity freshwater treatment. As the sodium levels of the tissue equilibrated with their higher saline environment the ratio of copper to sodium decreased significantly. This can again be described by the osmotic adjustment of the tissue to its environment. According to our ion exchange theory no alteration would occur to the copper levels of the gill tissue, which can be seen by the lack of significant statistical effect upon Cu concentrations in the gills. No significant change to the Cu/Na ratios occurred in carcasses from the 10 ppt treatment although a slight decrease was observed in the mean values as is consistent with our previous postulation.

The ratios of copper to magnesium were previously suggested as indicators of copper exposure, particularly in situation mixed metal exposure (Daglish et al. *in press)*. The Cu/Mg ratios in these experiments also showed significant effects over time, in all

three treatments. That the magnesium levels in the gill tissues of the carcasses increased in the two brackish treatments but were stable in the freshwater treatment is also reflected by the rate of change of the ratios. The freshwater treatment alters significantly between time 0 and 6 hours while there is no significant change until time 18 hours for the two brackish treatments. The Cu/Mg ratio may therefore provide a more useful indicator of copper induced mortality than Cu/Zn.

Macquarie Harbour

Muscle tissue copper levels

The levels of copper in the muscle tissue of salmonids from fish kill episodes in Macquarie Harbour were comparable to studies of copper levels in the axial muscle tissue of cultured channel catfish, *Ictalurus punctuatus,* from the Mississippi Delta, U.S.A. Mustafa and Mederios (1985) reported copper levels between 0.6 and 4.6 $\mu g.g^{-1}$ of muscle tissue with a mean value of 2.3 $\mu g.g^{-1}$ in animals taken from a catfish processing plant in the Mississippi Delta. Nettleton et al. (1990), who also examined catfish from a processing plant in the Mississippi Delta, found the copper levels of axial muscle tissue ranged from 0.3 - 1.2 $\mu g.g^{-1}$ with a mean value of 0.6 $\mu g.g^{-1}$. A third report of copper levels in muscle tissue of farmed and wild caught channel catfish, from the U.S. Department of Agriculture handbook (U.S.D.A., 1987) reported concentrations of 0.94 $\mu g.g^{-1}$. In the current study the range of values was 0.32 – 1.59 $\mu g.g^{-1}$ with a mean value of 0.84 $\mu g.g^{-1}$. This compares favourably with the U.S.D.A. report and is intermediate to the two other studies quoted.

The values for copper levels in muscle tissue from our field studies were higher than those of Griffin et al., (1997) who exposed channel catfish to three levels of waterborne copper (mean levels of dissolved copper of 220, 354 and 465 $\mu g.L^{-1}$) for ten weeks to examine the effects of remedial copper sulphate treatments in farm animals on the copper levels of axial muscle tissue in animals raised for human consumption. Their research showed no change in the recorded copper levels for any treatment over a 10-week period prompting the authors to conclude that remedial treatments of copper sulphate do not pose a health risk for consumers. Griffin et al., (1997) noted their treatments were at higher dosages and for longer duration than those normally used for remedial treatments. Given Macquarie Harbour animals may be exposed to continually fluctuating, but generally higher than normal, levels of environmental copper, and that our results compare favourably with those quoted above, it may be safe to assume the levels of copper present in Macquarie Harbour do not pose a health risk to consumers of animals farmed in the area. It is important to note however, that Macquarie Harbour has an unusual salinity profile and that the fish in the more saline environment may be expected to benefit from the ameliorative effects of the increased competition for binding sites at the gills.

Gill Copper Levels

The mean values of copper recorded in the gills of the animals from Macquarie Harbour were all higher than the levels recorded at time zero for the freshwater treatment and the 20 ppt treatment in the experimental data set. Gill copper levels for the 10 ppt treatment results are lower than all of the Macquarie Harbour results

except the lowest recorded case. While using the copper concentrations in the gills as indicators of exposure may be problematic due to the high levels of naturally occurring variation, these data demonstrate the animals from the fish kills recorded in Macquarie Harbour had levels of copper complexed to their gills that were comparable or higher than the levels of copper that induced moribundity in the laboratory trials. This strongly suggests that copper may have had a causative role in these fish kills. However, the animals from the laboratory trials were previously unexposed to elevated ambient copper levels. Animals from Macquarie Harbour may have been exposed *a priori* to elevated levels of copper in the water column throughout the period of their husbandry. The phenomenon of acclimation, where an animal is more tolerant of a toxicant after a previous non-lethal exposure is well documented for copper toxicity (Dixon and Sprague, 1981b; Grosell et al., 1997; McDonald and Wood, 1993). However some studies of chronic exposure did not find any change in copper accumulation (Dixon and Sprague, 1981a; Lauren and McDonald, 1987b).

Metal Ratios

The Cu/Zn ratios in the Macquarie Harbour animals were considerably lower than the values recorded for the laboratory trials and also much lower than in previous studies that examined Cu/Zn ratios as biomarkers in freshwater (Carbonell and Tarazona, 1993; Daglish and Nowak, 2002; Daglish et al. *in press*). This is due to the high levels of zinc recorded in the gills of the Macquarie Harbour animals, which were also substantially higher than the levels recorded in any of the laboratory trials or

those found in the literature, for both exposed and unexposed animals (Galvez et al. 1998; Mount 1964).

The unusually high levels of zinc in the gills of the Macquarie Harbour animals are difficult to explain, as zinc does not accumulate significantly at the gill surface (Sorensen, 1991) in the same manner as copper, but passes through the calcium channels, as a calcium analogue (Markich and Jeffree, 1994). While it is well documented that Macquarie Harbour has suffered from chronic metal pollution (Carpenter et al., 1991; Koehnken, 1996; Stauber et al., 1996) there are no records of zinc levels covering the time period of the fish kill episodes and further investigations would be required to understand the causes of the levels of zinc recorded in the gills of the field animals. However, in contrast to the laboratory trials these data suggest Cu/Zn ratios may not be applicable as biomarker in animals acclimated to brackish waters. Alternatively, the animals may have died of other causes. However, the significant differences between the laboratory and the field studies demonstrate the need for a detailed understanding of the local environmental conditions when assessing the data gathered from the field.

In conclusion, gill copper loads of rainbow trout at the time of death were greatest in animals in an isotonic environment and lowest in animals in a hypertonic environment. We proposed an 'ion exchange' model to account for this observation. While the BLM demonstrates mortality may be predicted from the copper load of the gills in freshwater animals, the current model may require revision to be suitable for

isotonic and hypertonic environments where different physiological mechanisms are involved in ion regulation. Also gill metal ratios may be useful as biomarkers in the few hours immediately after death in freshwater animals. However, by 6 hours post-mortality the osmotic adjustment of the tissue to the environment limits the usefulness of this diagnostic tool. In waters of higher salinities the osmotic adjustment is slower and the metal ratios may be practical for up to 18 hours. Finally, examination of muscle tissue from farmed animals from fish kills which occurred in a brackish water body known to have regularly elevated copper levels did not pose any apparent heath risk to consumers. They did, however, record higher zinc levels in the gills than expected from the laboratory trials and other literature. This precluded the utility of the Cu/Zn ratios as a diagnostic tool for the cause of the deaths and highlighted the need for knowledge of local water quality parameters in fish kill episodes.

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Chapter 5 Dissolved organic carbon in brackish waters reduces copper binding to the gills of rainbow trout (Oncorhynchus mykiss).

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Abstract

Rainbow trout were exposed to nominal additions of 0, 20, 40, 80, 200 and 400 μgL^{-1} of waterborne copper in brackish waters of 14 ppt salinity containing 3 different levels of dissolved organic carbon (2.0, 4.7 and 22.8 mgL^{-1}). The experiments were run for 12 hours. Total and ASV labile copper were measured in the treatments and the concentrations of Cu, Zn, Ca, Mg and Na in the gills of the animals were measured at the end of the trials. Gill Cu was significantly different in all treatments indicating the capacity for DOC to prevent Cu binding to the gill epithelium of the fish in brackish waters. ASV-labile copper showed a bi-phasic relationship to total copper measurements in the two lower DOC treatments indicating a saturation of binding sites prior to an increase in 'free' copper. The high DOC treatment was monophasic in its relationship to total copper suggesting the binding capacity of the DOC was not saturated. ASV-labile copper did not adequately describe the binding of the bioavailable portion of the copper to the fish gills.

Introduction

To date there has been little research examining the effects of elevated ambient copper concentrations on teleosts in marine or brackish waters. Most studies have investigated the effects of metals upon branchial ion regulation and plasma electrolyte concentrations (Stagg and Shuttleworth 1982a; Stagg and Shuttleworth 1982b; Taylor et al. 1996; Wilson and Taylor 1993a; Wilson and Taylor 1993b), the effects of copper-treated pens in fish farms on animals produced for human consumption (Lewis and Metaxas 1991; Peterson et al. 1991) and levels of metals in the tissues of natural populations of animals that may be affected by discharges from industry. (Lewis et al. 2002; Powell and Powell 2001; Wong et al. 2000) Reports from Nowak and Duda (1996) and Stauber et al. (1996) investigating copper toxicity in Macquarie Harbour, in western Tasmania, and other reports from Westerlund et al. (1998) and Kurilenko et al. (2002) are among the few investigations of copper toxicity to teleosts in marine and brackish environments.

In contrast, the toxicity of copper to teleosts in freshwater has been well elucidated. Mortality results from the inhibition of the active transport of sodium across the gills caused by competitive interactions with the copper ion. The depletion of plasma sodium is exacerbated by losses across the inter-cellular tight junctions within the gills due to increased ionic permeability (Lauren and McDonald 1985). Copper toxicity is more closely related to the concentration of the free copper ion, Cu^{2+}, in the water column than total dissolved copper (Campbell 1995). However, the Biotic Ligand Model (BLM) has also determined that mortality is dependent on the total

amount of copper bound at the gills, rather than the concentration of either total copper or free copper in the water column. (Di Toro et al. 2001; Paquin et al. 2002; Santore et al. 2001)

Speciation of copper in natural waters, and therefore the proportion of bioavailable or free copper, may be affected by inorganic factors that alter chemical speciation such as pH, alkalinity and hardness. Alternatively, organic factors may reduce the bioavailable fraction of copper through complexation of the copper by dissolved organic matter or adsorption to suspended solids and particulate matter in the water column (Erickson et al. 1996; Hollis et al. 1997; Lauren and McDonald 1986; McGeer et al. 2002; Pagenkopf 1983; Playle et al. 1993a; Playle et al. 1992). Dissolved organic carbons (DOC) such as humic and tannic acids may be a prominent factor in reducing copper toxicity in natural water bodies and have received significant attention in recent laboratory based studies of copper toxicity in freshwater (Erickson et al. 1996; Hollis et al. 1997; Lorenzo et al. 2002; McGeer et al. 2002; Playle et al. 1993a). It is generally agreed that DOC has the ability to complex copper thereby reducing copper toxicity to teleosts by decreasing the concentration of the toxic Cu ion that may bind to the fish's gills.

Metal ions in seawater are maintained in their ionic state unless modifying organic or inorganic factors are present to either complex or adsorb the metals. The higher ionic composition of seawater should result in greater competition between the different ions for binding sites at the gills (Pagenkopf 1983; Playle 1998). This may ameliorate

metal toxicity so that heavy metals are less toxic in seawater than in freshwater at the same concentrations. Experimentally determined LC50s for copper in marine studies have been higher than in freshwater studies (Eisler and Gardner 1973; Taylor et al. 1985).

As copper is a disruptor of sodium branchial regulation it may still have a deleterious effect upon branchial ion regulation in marine and euryhaline teleosts (Taylor et al. 1996; Wilson and Taylor 1993a; Wilson and Taylor 1993b). In a saline or brackish environment hyper-osmotic to the animal's internal milieu, it is necessary for teleosts to prevent excess sodium from the environment moving across the gill epithelium into the animal. Sodium regulation by the gills is principally to extrude excess sodium from the animal's internal environment, i.e. opposite to that of freshwater animals (Evans 1993; Karnaky Jr 1998). Daglish et al. (submitted for publication) have therefore argued the current BLM may not incorporate the different physiological requirements of euryhaline and marine fish.

As it is now established that dissolved organic carbon will reduce the toxicity of copper in freshwater by reducing the concentration of free copper available to bind to the gill epithelium, it was decided to investigate if a similar effect would be found in brackish waters. Macquarie Harbour on the western coast of Tasmania is high in naturally occurring DOC and water from the harbour were used as part of this study to provide both brackish waters with DOC as well as a field based element to the study.

Macquarie Harbour

Macquarie Harbour, located on the western coast of Tasmania, is approximately 32 km long and 8 km wide with an area of 276 km^2 and a mean depth of approximately 20 m. There is a narrow opening to the Southern Ocean at the north-western corner of the harbour. Fresh water high in dissolved organic carbons from the dense riparian vegetation flows into the harbour from the King River in the north-eastern area of the harbour and the Gordon-Franklin River system in the south-eastern section. Tidal movements of the harbour are generally less than 1m.

The water of Macquarie Harbour is distinctly stratified into two layers. The surface waters of the harbour, defined as 0 - 5 m, are characterised by highly variable salinities and high levels of organic carbons in the form of tannic and humic substances giving the surface waters a distinctive brown "tea-like" appearance. The remainder of the water column is saline. The variability of the surface water's salinity and organic content is mainly due to rainfall, changes in the flow of freshwater from the two river systems and mixing by wind (Carpenter et al. 1991; Koehnken 1996). Waters collected from Macquarie Harbour for this study were taken from the surface layer.

The aim of this experiment was to examine the effect of different levels of dissolved organic carbon on the capacity of copper to bind to the gills of rainbow trout in

brackish waters and to determine if increased levels of DOC will reduce the amount of bound copper at the gill epithelium.

Methods and materials

Animals

Rainbow trout (n = 144; mean wt 42.5g; SD = 12.0 g) were obtained from the Aquaculture Centre, University of Tasmania. The animals were acclimated to the experimental water salinity in a 4 000 L recirculating tank supplied with town water supplemented by the addition of filtered seawater. Water quality parameters were: Salinity = 14 ppt, T = 13.2 ± 0.2 °C, pH = 7.6 ± 0.1, D.O. = 8.9 ± 0.4 mgL^{-1}, NO_2^- < 0.1 mgL^{-1}, NO_3^- < 0. 02 mgL^{-1}. Animals were transferred to 60 L tanks for the experimental trials.

Experimental Design

Three trials were run, the first using 'clean' brackish water (CBW) prepared from a mixture of filtered seawater and local town water. The second trial (MH) was run using water from Macquarie Harbour, Tasmania, which had low levels of naturally occurring DOC. Water for the last trial (HHA) was prepared by soaking button grass (*Gymnoschoenus sphaerocephalus)* in water from Macquarie Harbour for 4 days by which time the water had turned a deep 'tea' colour from the organic carbons being released. Button grass was used due to mimic the creation of the tannins in the rivers feeding Macquarie Harbour, thereby producing organic carbons equivalent to those found occurring naturally in Macquarie Harbour. Six concentrations of copper, nominally 0, 20, 40, 80, 200 and 400 μgL^{-1} were run in the CBW and MH trials while 5 treatments, nominally 0, 20, 80, 200 and 400 μgL^{-1}, were run in the HHA trial. Total measured copper and Anodic Stripping Voltammetry (ASV) copper

concentrations are given in table 1. Each trial was run for 12 hours as previous work had shown gill binding sites for copper were saturated by this period of time (Daglish, unpublished data).

The experiments were conducted in 60 L plastic tanks with 8 fish randomly assigned to each tank. Water samples were taken from each copper concentration before and after each experiment and conditions for each of the trials were as follows: Group 1 (CBW, n = 12)); T = 17.5 ± 0.1 °C, D.O. = 9.90 ± 0.30 mgL^{-1}, DOC = 2.0 ± 0.0 mgL^{-1}, Total NH_3 < 0.1 mgL^{-1}, NO_2 <0.1 mgL^{-1}, NO_3 <0.02; Group 2 (MH, n = 12); T = 18.1 ± 0.3 °C, D.O. = 8.9 ± 0.3 mgL^{-1}, DOC = 4.7 ± 0.5 mgL^{-1}, Total NH_3 < 0.1 mgL^{-1}, NO_2 <0.1 mgL^{-1}, NO_3 <0.1; Group 3 (HHA, n = 10); T = 17.6 ± 0.2 °C, D.O. = 9.7 ± 0.2 mgL^{-1}, DOC = 22.8 ± 0.4 mgL^{-1}, Total NH_3 < 0.1 mgL^{-1}, NO_2 <0.1 mgL^{-1}, NO_3 <0.02.

At the end of the trials animals were euthanased by adding 1 mL of clove oil to 8 litres of water from the treatment tank. The animals were then weighed and the gills removed. Gills were placed immediately on ice prior to being stored at -20°C until being prepared for metal analysis.

DOC Analysis

Analysis of DOC was conducted by Australian Government Analytical Laboratories (AGAL). Samples were collected in 200 mL HDPE containers and sent immediately upon collection to AGAL. The concentrations of Total Organic Carbon (TOC) and

Dissolved Inorganic Carbon (DIC) were determined using a High Temperature Dohrman DC-190 Elemental Analyser. The concentration of DOC is equal to TOC – TIC.

Metals Analysis

Total metal concentrations in the experimental treatments and the gill filaments were determined using a Varian Spectra AA 300 Atomic Absorption Spectrometer (AAS). The 'free' copper ion levels of each treatment's water were measured by Anodic Stripping Voltammetry (ASV copper) using a Metrohm 646 VA Processor with a hanging drop mercury electrode.

Water samples were collected at the start and end of each trial in acid-washed 200 mL high density polyethylene containers. ASV copper analysis was performed immediately after the collection of the samples. Twenty millilitres of each sample was pipetted into a clean glass sample cell for analysis. Samples were purged with nitrogen for 5 seconds before deposition at -1.15 V (*vs.* saturated calomel) for 60 seconds. The potential was scanned at $10mVsec^{-1}$ to 0.140 V. A standard curve of peak height *vs.* free copper was constructed for copper additions in filtered seawater ranging from 0 to 400 μgL^{-1} $[Cu^{2+}]$ ($r^2 = 0.981$).

The $[Cu^{2+}]$ of the various treatments were determined by their peak height from the standard curve of the seawater plot. The retained portion of the water samples were

then acidified with a few drops of concentrated HNO_3 for total copper analysis. AAS analysis of the samples was conducted within 24 hours of collection.

Gill filaments were dissected from the arches and digested in 5 mL of concentrated HNO_3 in Teflon digest vessels in a CEM MDS-2000 microwave digester (2.5 min at 630 W; 30 mins at 500 W). Between 50 and 200 mg of filamentous tissue was used for each digest. Digest vessels and glassware were washed in 10% HNO_3 prior to use. Copper, zinc and magnesium were analysed by atomic absorbance using an air/acetylene flame. Calcium analysis was performed using a nitrous oxide/acetylene flame and sodium was analysed by emission spectrometry with an air/acetylene flame. All analyses were performed using a Varian AA 300 Atomic Absorption Spectrometer. Plasma samples for calcium, magnesium and sodium were diluted 1:20 with deionised water prior to analysis. Standards were run prior to and after each set of analyses to monitor possible drift during analysis. Samples of the gills were spiked with 1 mgL^{-1} of Cu^{2+} and recovery rates were determined. These ranged from 98% to 106%. National Institute of Standards and Technology bovine liver samples (NIST 1577b) were run to verify the laboratory methods. All data presented are for wet weight (w/w) of tissue.

Statistical Analysis

The concentrations of the various metals in the gills were analysed in each water quality group by multivariate analysis to determine if there were significant differences between treatments. Differences between treatments were considered

significant at the $\alpha = 0.05$ level using Pillai's Trace. All data were tested for equality of error variance using Levene's Test. Normality of data was confirmed using pp plots and histograms of the variables.

Multivariate analysis was also conducted for the concentration of the various gill metals between groups, based on the concentration of total copper in the water column. Due to variations in the total measured levels of copper in the different water quality groups, a low, mid and high copper concentration was chosen for comparisons between each group. These were treatments 1, 3 and 5, being 29.5 μgL^{-1}, 169.7 μgL^{-1} and 295.3 μgL^{-1} from group CBW; treatments Ctrl, 4 and 5, being 45.7 μgL^{-1}, 154.6 μgL^{-1} and 271.9 μgL^{-1} from group MH; and treatments 1, 4 and 5, being 39.7 μgL^{-1}, 167.9 μgL^{-1} and 318.6 μgL^{-1} from group HHA (Table 1).

The levels of ASV copper were determined as a percentage of the total copper for all treatments in each of the three water quality categories. Univariate analysis of the three groups was performed to determine if significantly different levels of free copper were available between the groups.

Post hoc analyses were performed using Tukey's test. All statistical analyses were performed using the software package SPSS v 10.0.

Table 5.1 Total and ASV Cu levels and the percentage of ASV Cu against total Cu for all experimental treatments.

Trial	Treatment	Total [Cu] μgL^{-1}	ASV [Cu] μgL^{-1}	ASV/Total Cu
CBH	Ctrl	15.4	12.1	0.79
	1	29.5	22.9	0.77
	2	83.2	35.8	0.43
	3	169.7	76.0	0.45
	4	295.3	181.3	0.61
	5	420.8	323.7	0.77
MH	Ctrl	45.7	13.8	0.30
	1	67.4	20.0	0.30
	2	88.7	25.2	0.28
	3	95.5	40.0	0.42
	4	154.6	53.1	0.34
	5	271.9	141.6	0.52
HHA	Ctrl	20.3	6.2	0.31
	1	39.7	15.2	0.38
	3	76.7	21.9	0.29
	4	167.9	37.6	0.22
	5	318.6	80.1	0.25

Results

Between Subject Analysis

Multivariate analysis with DOC levels as the fixed factor and the gill concentrations of copper, zinc, calcium, magnesium and sodium as the dependent variables were found to be significant as determined by Pillai's Trace ($F = 12.025$, $d.f = 134$, $p < 0.001$).

Post hoc analysis using Tukey's Test differentiated between all three groups for copper concentrations in the gills. The highest copper concentrations in the gills were found in the group CBW being 1.72 ± 1.17 μgg^{-1} while the lowest concentration of 0.34 ± 0.16 μgg^{-1} was in the group HHA. The group MH had a median value of 0.92 ± 0.64 μgg^{-1} of gill copper (Fig 1).

Calcium similarly separated into three distinct subsets upon *post hoc* analysis. The highest concentration of calcium in the gill tissue, of 449.9 ± 152.7 μgg^{-1}, was found in the group CBW, while the lowest concentration of 231.6 ± 65.3 μgg^{-1} was in the group HHA. The group MH had a median value of 359.9 ± 155.0 μgg^{-1} of gill calcium

For the metals zinc, magnesium and sodium, *post hoc* analysis separated the groups into two subsets. The highest zinc concentration of 8.50 ± 3.8 μgg^{-1} were in the gills of the animals from the group CBW, which was significantly different to the lowest concentration of gill zinc of 5.54 ± 4.2 μgg^{-1} in the group HHA. The gill zinc

concentration of MH, 7.85 ± 3.1 $\mu g g^{-1}$, was not significantly different to either of the other groups.

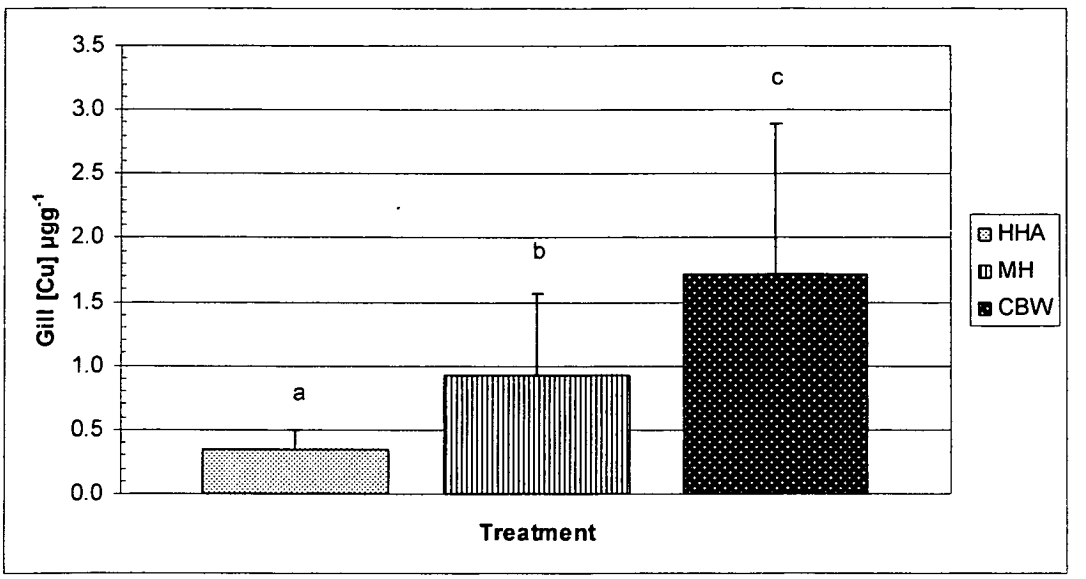

Fig 5.1 Mean and standard deviations of Cu concentrations in the gills of rainbow trout exposed to elevated ambient copper in brackish waters with three differing concentrations of dissolved organic carbon. DOC concentrations in the treatments were; CBW = 2.0 ± 0.0 mgL^{-1}, MH = 4.7 ± 0.5 mgL^{-1}, DOC = 22.8 ± 0.4 mgL^{-1}. Homogenous subsets are indicated by lower case letters.

The highest magnesium concentration was found in the gills of the MH group; 54.67 ± 11.4 μgg^{-1}. This was significantly greater than the lowest concentration of 37.59 ± 9.8 μgg^{-1} recorded in the gills of the animals from the group HHA. The concentration of magnesium in the third group, CBW, was 45.56 ± 19.3 μgg^{-1} which was not significantly different to either other group.

The highest sodium concentration of 349.9 ± 52 μgg^{-1} was recorded in the gills of the group MH. This was significantly greater than either of the other groups, the concentrations being 254.5 ± 62 μgg^{-1} for the group CBW and 255.9 ± 38 μgg^{-1} for the group HHA.

ASV Cu

A significant difference was observed between the three groups for the fraction of ASV copper *vs* total copper ($F = 13.705$, d.f. = 2, $p < 0.001$). *Post hoc* analysis using Tukey's test separated the CBW treatment from the HHA and MH treatments. The mean percentage of the ASV Cu fraction for the CBW was 63.7 ± 16.6% whereas the fraction of ASV Cu in the MH and the HHA treatments were 36.0 ± 9.3% and 29.0 ± 6.1% respectively (Fig 2).

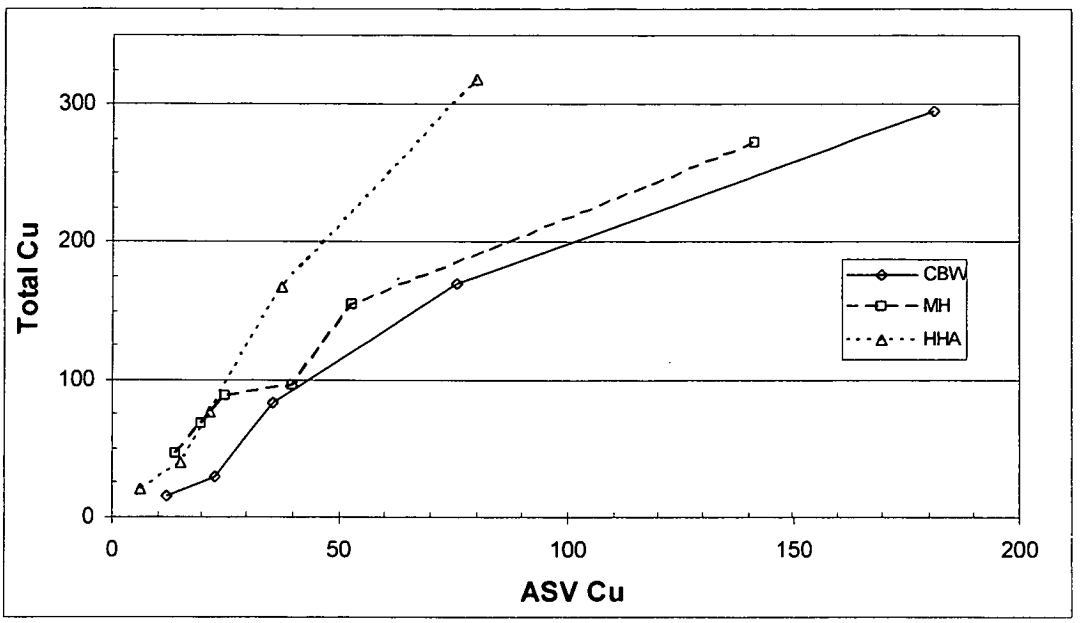

Fig 5.2 Total measured Cu *vs* ASV-labile Cu in waters with 3 different levels of DOC: CBW, DOC = 2.0 ± 0.0 mgL^{-1}; MH, DOC = 4.7 ± 0.5 mgL^{-1}; HHA, DOC = 22.8 ± 0.4 mgL^{-1}. Single experimental readings only.

With-in Subject Analysis

Group 1 – 'Clean' Brackish Water (CBW)

After 12 hours exposure to 5 levels of waterborne copper in clean brackish water at 14 ppt salinity, multivariate analysis showed significant differences in the copper residues of the gill filaments of the rainbow trout ($F = 1.806$, d.f. = 25, $p = 0.014$). The highest reading of copper residues was in treatment 5, the mean value being 3.07 ± 1.3 $\mu g.g^{-1}$ of tissue while the lowest recorded mean value was 0.86 ± 0.5 $\mu g.g^{-1}$ of tissue in the control treatment. *Post hoc* analysis using Tukey's Test separated the gill copper concentrations into two groups with the control group and treatment 1 being autonomous of treatment 5 (Fig 3).

No other significant differences were recorded between the different treatments for any of the other metal concentrations measured in the gill filaments. Mean values and standard deviations were as follows: Zn 8.07 ± 3.8 $\mu g.g^{-1}$ of tissue; Ca, 422.4 ± 146 $\mu g.g^{-1}$ of tissue; Mg, 44.6 ± 14 $\mu g.g^{-1}$ of tissue; and Na, 248.9 ± 61 $\mu g.g^{-1}$ of tissue.

Group 2 – Macquarie Harbour water (MH)

After 12 hours exposure to 5 levels of waterborne copper in water from Macquarie Harbour at 14 ppt salinity and a mean level of 4.7 ± 0.5 mgL^{-1} of naturally occurring DOC, multivariate analysis showed significant differences in the copper residues of the gill filaments of the rainbow trout ($F = 2.624$, d.f. = 25, $p < 0.001$). The highest reading of copper residues was in treatment 5, the mean value being 1.53 ± 0.36 $\mu g.g^{-1}$ of tissue while the lowest recorded mean value 0.17 ± 0.1 $\mu g.g^{-1}$ of tissue was

recorded in treatment 1.

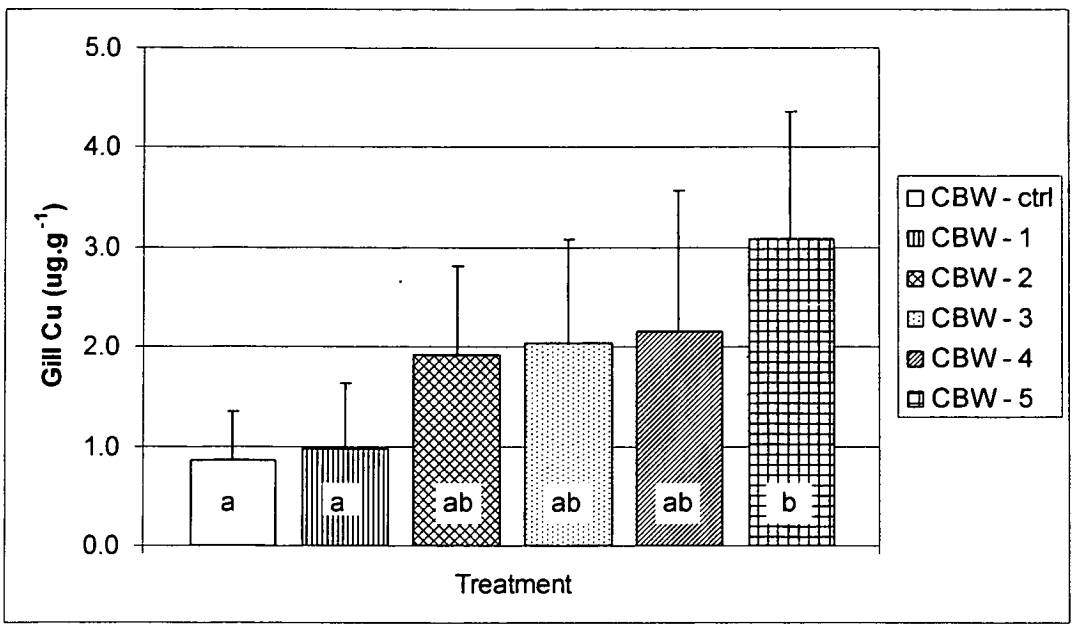

Fig 5.3 Mean and standard deviation of Cu concentrations in the gills of rainbow trout exposed to elevated ambient copper in 'clean' brackish water (CBW). Homogenous subsets are indicated by lower case letters.

No other significant differences were recorded between the different treatments for any of the other metals measured in the gill filaments. Mean values and standard deviations for the values of all the combined treatments were as follows: Zn 8.11 ± 4.1 $\mu g.g^{-1}$ of tissue; Ca, 357.7 ± 153 $\mu g.g^{-1}$ of tissue; Mg, 53.5 ± 11 $\mu g.g^{-1}$ of tissue; and Na, 341.1 ± 51 $\mu g.g^{-1}$ of tissue.

Post hoc analysis using Tukey's Test separated the copper levels into three subsets with the control group and treatments 1 and 2 being autonomous of treatment 4. Treatment 5 also was significantly different to treatment 4 (Fig 4).

Group 3 – High Humic Acid (HHA)

After 12 hours exposure to 5 levels of waterborne copper in brackish water at 14 ppt salinity in which button grass was soaked to increase the mean level of DOC to 22.8 ± 0.4 mgL^{-1}, multivariate analysis showed significant differences in the copper residues of the gill filaments of the rainbow trout ($F = 1.719$, d.f. = 25, $p < 0.036$). The highest gill copper concentration was in treatment 5, the mean value being 0.397 ± 0.13 $\mu g.g^{-1}$ of tissue while the control group of animals recorded the lowest mean value 0.192 ± 0.06 $\mu g.g^{-1}$ of tissue.

No other significant differences were recorded between the different treatments for any of the other metals measured in the gill filaments. Mean values and standard deviations for the values of all the combined treatments were as follows: Zn 5.06 ± 3.9 $\mu g.g^{-1}$ of tissue; Ca, 224.2 ± 61 $\mu g.g^{-1}$ of tissue; Mg, 36.6 ± 8 $\mu g.g^{-1}$ of tissue; and

Na, 262.4 ± 39 $\mu g.g^{-1}$ of tissue. *Post hoc* analysis using Tukey's Test separated the copper levels into two subsets with the control group being autonomous of treatment 5 (Fig 5).

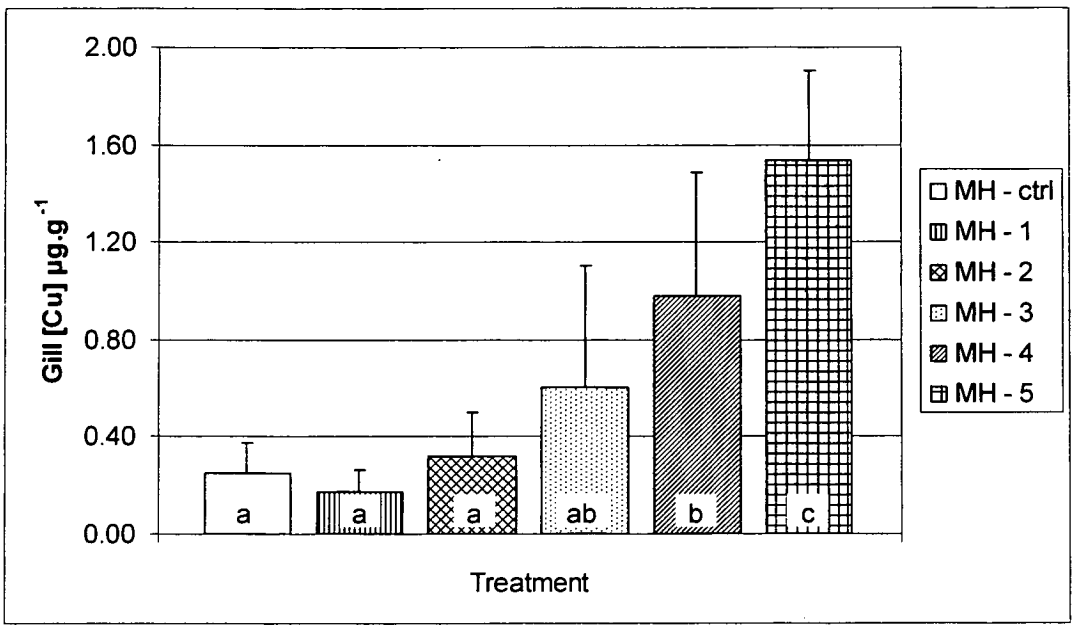

Fig 5.4 Mean and standard deviations of Cu concentrations in the gills of rainbow trout exposed to elevated ambient copper in Macquarie Harbour Water (MH). Homogenous subsets are indicated by lower case letters.

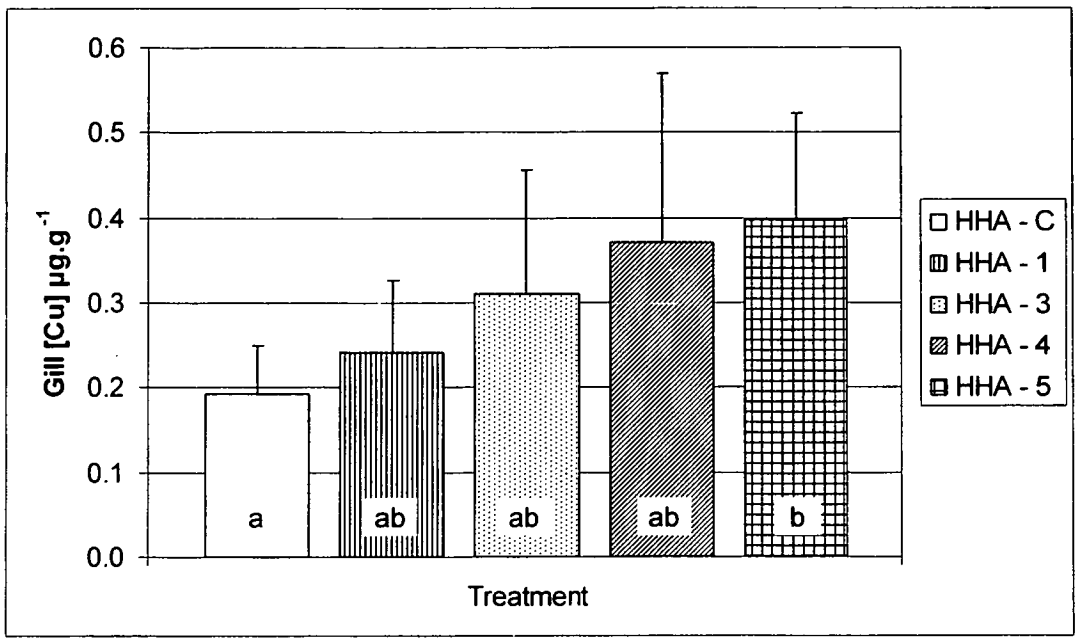

Fig 5.5 Mean and standard deviations of Cu concentrations in the gills of rainbow trout exposed to elevated ambient copper in brackish water with high levels of dissolved organic carbon (DOC). Homogenous subsets are indicated by lower case letters.

Discussion

The results of these experiments clearly demonstrate that increased levels of dissolved organic carbon will reduce the amount of copper that binds to the gill epithelium of rainbow trout in brackish waters. This reduction may be attributed to a reduction in the fraction of bio-available copper i.e. free Cu^{2+} ion.

For the treatments CBW and MH it can be seen from Fig 2 that the relationship between the two forms of measured copper is biphasic whereas the treatment HHA is essentially monophasic. The biphasic shape in the first two treatments suggests the adsorption or binding of copper by complexing agents in the water column, prior to a saturation of the available binding agents. This occurs to a lesser degree in the treatment CBW and may be due to the presence of secreted proteins from the animals such as mucous, and also the formation of various copper species with in the brackish matrix. That the proportion of bound copper is less in the MH treatment suggests the complexing capacity of the naturally occurring dissolved organic carbons. The monophasic nature of the HHA treatment indicates the high levels of dissolved organic carbons are yet to reach their saturation point.

In the treatment MH there is a sharp increase in the proportion of ASV copper from 25 to 40 μgL^{-1}. This suggests the possibility of different complexing sites with differing binding affinities within the DOC. As dissolved organic carbon is a collective term for many different long-chain organic acids that are susceptible to degradation, it is feasible various binding potentials exist among the organic acid

components. In titrations of Cu against dissolved organic matter isolated from wastewaters, Ma et al. (2001) isolated three separate fractions of organic carbons, being humic acid, fulvic acid and a hydrophilic component. The binding characteristics of these fractions were based on a two-site discrete ligand model. The primary binding sites were less stable, interacted rapidly with copper and were associated with carboxylic groups in the DOC. The secondary binding sites were smaller and had a higher conditional stability constant. Ma et al. (2001) also found the hydrophilic portion of the DOC had different binding characteristics to the humic and fulvic acids, and proposed the predominating Cu-binding functional groups may be different between these portions. These findings suggest the rapid shift in the ASV-labile Cu in the MH treatment may be due to the copper binding to different sites of the dissolved organic carbon. However, no similar shift occurred in the HHA treatment. As there was a five-fold increase in the level of DOC in the HHA treatment, the binding sites may not be saturated. Also, the distance between measured points could easily mask such a shift. Alternatively the sudden change in the MH treatment may result from an anomaly within the data collected.

Regression analysis of total copper to gill copper provided a better indication of gill binding capacity than ASV-labile copper to gill copper in all three treatments. This is a surprising result given the obvious decrease in the proportion of 'free' copper as measured by ASV methods. A potentially confounding aspect of these experiments is the use of a static experimental system. As fish are continually producing and sloughing off mucus there is the potential for the glyco-protein mucus matrix in the

water column to affect the copper speciation in the experimental system. We may conjecture that mucus production would bind some fraction of the total copper present in the water column but it is difficult to predict the exact effect of the mucus upon the dynamic interchanges between species of copper in a brackish environment. It is, however, notable that there are numerous potential interactions between the DOC, the gill epithelium, mucus in the water column, the fish's bodies and the sides of the containers, all of which will adsorb some fraction of the waterborne copper and act as a pool of potentially exchangeable copper within the system. A more simple solution would be to use a flow-through system for experiments requiring the measurement of the subtle chemical states of copper speciation, thereby removing excess mucous as from the system as it is produced. This may not always be possible, as in these experiments, due to the logistics of transporting large volumes of natural waters.

Stauber et al. (1996) and Eriksen et al. (2001) both found ASV copper overestimated the toxicity of copper to the algae *Nitzschia closterium* in experiments conducted in Macquarie Harbour waters. Stauber et al. (1996) discussed the probability that the ASV-labile proportion of copper also includes inorganic and organic complexes that can disassociate at the ASV electrode and states that the ASV-labile portion of the copper is not necessarily comparable to the bio-available fraction of copper in the water column. This argument also highlights the ambiguity of the term 'bioavailable'. The binding constants for different biotic forms are not likely to be comparable; for example, the binding constant for copper to the external wall of an algal cell and the

epithelium of fish gills. While ionic copper is assumed to be bioavailable due to its unbound or 'free' status other copper species may also be available to marine life. However, our results demonstrate that ASV-labile copper is not a suitable indicator of the bioavailable fraction of water-borne copper for rainbow trout in brackish waters.

Of the other metals measured in the gills of the rainbow trout, calcium and zinc both followed the same pattern as copper. Their concentrations were highest in the treatment CBW and lowest in the treatment HHA. The zinc concentrations were significantly different only between the HHA treatment and the CBW treatment while the calcium levels were significantly different between all three groups. This may be a reflection of a reduced availability of the metals due to binding to the organic carbons within the system. While this is attributing an enormous capacity for complexation to these dissolved organic carbons, the work by Ma et al. (2001) indicated such levels of complexing are not unfeasible. It should also be noted the level of dissolved organic carbons in the treatment HHA, (22.8 mgL^{-1}) is substantial.

The effects upon sodium and magnesium also appear anti-intuitive and this argues against a generalised diminution of metal ions due to complexing by high DOC levels. An alternative explanation is that competitive interactions between the various ions for DOC binding sites follow a hierarchical pattern where Cu, Ca and Zn bind with greater affinities than do Na and Mg. As Na is a mono-valent ion it is most likely to behave differently to the other ions. Also, the term, 'dissolved organic carbon' is a catch-all phrase that does not differentiate between various components

of the material, it is therefore difficult to make any generalisations about the binding capacity of the DOC, other than it has a strong complexing capacity.

In conclusion, this work demonstrated that high levels of DOC will reduce the binding of copper to the gills of fish in brackish waters. This will reduce the toxicity of copper to brackish fish although further work is needed to quantify the physiological and toxicological effects of copper under various DOC regimes. While the reduction of the bound copper may be attributable to a reduction of free copper in the water column, measurements of ASV-labile copper do not adequately describe the fraction of copper that is bio-available to the fish. Additionally, the MH treatment suggests the DOC may bind copper at multiple sites although this was not substantiated in the HHA treatment. The lack of corroborating evidence in the HHA treatment may be due to the primary binding site having yet to be saturated with the copper.

Acknowledgments

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Chapter 6 General Discussion

The first set of experiments examined copper accumulation in the gills, liver and plasma of rainbow trout exposed to acute concentrations of waterborne copper. We also examined the ratios of copper to zinc in the gills and liver and the ratio of $gills_{[Cu]}$ to $liver_{[Cu]}$. These experiments provided the starting point of our investigations into biomarkers for teleosts exposed to acute, short-term copper exposure.

The main findings were: the gills provided a better indication of copper exposure under acute short-term exposures than the liver, copper/zinc ratios in the gills indicated short-term exposure to elevated waterborne concentrations of copper, and circulating levels of copper in the plasma did not affect gill or liver copper concentrations in exposed animals.

In the light of the current literature the first of the findings would now appear to be well substantiated. The development of the BLM has increased the understanding of the gills as the target of toxicity for metal, notably copper, and focused research on gill-metal interactions. The proposal and demonstration of a threshold limit of copper binding at the gills by which it is possible to predict toxicity (Meyer et al. 1999) is, perhaps, the most telling indication of this concept. While my research does not include the sophisticated approach of the BLM, it contributes to the literature detailing the accumulation of copper at the gills as a primary means by which to recognise metal intoxication or exposure has occurred.

Additionally, a recent evaluation of chronic indicators of copper exposure rated gill accumulation of copper on the gills as a better indicator of exposure than growth, sprint performance, plasma electrolyte loss or acclimation (Taylor et al. 2000). The binding characteristics of the gills were considered a better chronic indicator than gill accumulation. At low levels of exposure this method required the use of Cu^{64} radio-tracers which may not be feasible in remote areas or in many analytical laboratories. However for my experiments under acute exposure regimes, the more coarse measure of total gill copper compared against gill zinc was sufficient to indicate exposure. Although it is difficult to compare chronic and acute exposure regimes, it is worthwhile to note that gill accumulation is a potential indicator of exposure under either of these conditions.

The second finding, that gill copper/zinc ratios may be an indicator of copper exposure, gave an initial validation to the proposal of Carbonell and Tarazona (1993) that such a ratio may be suitable when control animals are unavailable. This provided sufficient impetus to continue this line of research as was seen from chapters 3 and 4. The principle advantages of using the ratios was their ease of use for chemists or technicians at mine sites in remote areas to monitor local fish populations without the need to use complex biological assays.

The last significant result of chapter two, that circulating plasma levels did not affect the highly vascularised organs of the gills or the liver has also been validated, albeit indirectly, in recent publications. The role of the liver as the principle organ of copper

regulation has been clearly demonstrated (Grosell 2001; Grosell et al. 1998) as has the interaction between the gills and the liver in regulating copper uptake and clearance (Kamunde et al. 2002). The overall picture to emerge is one of tight regulation of plasma copper levels which supports the finding of plasma copper not affecting either gill or liver total copper concentrations.

This is in contrast to work by Pelgrom et al. (1995b) where exposure to concentrations of 50, 100 and 200 μg L^{-1} of total measured copper all increased copper concentrations in the plasma of tilapia. This may be due to species differences as the work of Grosell et al. (1998), Grosell (2001) and Kamunde et al. (2002) was performed with rainbow trout. Salmonoids are generally considered as 'metal-sensitive' and it would therefore be of interest to conduct experiments on copper metabolism on an alternative and more robust species to verify the results garnered thus far.

The second set of experiments, detailed in chapter 3, exposed rainbow trout to various concentrations of waterborne copper and zinc for 12 hours. The concentrations of Cu. Zn, Ca, Mg and Na in the gills of the animals were determined by AAS. It was deemed necessary to test the putative biomarker under conditions of copper and zinc exposure to determine if this would affect the zinc concentrations in the gills. These experiments highlighted the potential of Cu/Na ratios as a more effective biomarker than Cu/Zn ratios. The ratio of Cu/Mg also was noted as being significantly different under the exposure regimes used. A final finding documented

in the experiments was the intriguing reduction of plasma calcium in the high copper treatment. This reduction of plasma calcium and a possible cause has been discussed earlier in chapter 3.

The differences in Cu/Na ratios was due to both an increase in the gill copper concentrations and a reduction in the concentration of sodium in the gill tissue although sodium levels in the plasma were not significantly affected. Copper transport across the gill epithelium is now known to have a 'sodium-sensitive' component that correlates to the apical sodium channel (Grosell and Wood 2002). Historically, copper disruption of sodium regulation at the gills has been attributed to an inhibition of the Na^+/K^+- ATPase transport enzyme embedded in the baso-lateral membrane of the gills (Lauren and McDonald 1985). Consequently, concentrations of sodium in the plasma are also affected. However in this study the concentration of sodium in the tissue decreased whereas no effect was seen on plasma sodium levels.

This finding would be consistent with disruption at the apical membrane of the gills occurring prior to any subsequent interruption of sodium transport at the baso-lateral membrane. As Grosell and Wood (2002) noted, there is a time dependent component to the effects of copper upon the disruption of sodium transport, as well as a relationship to ambient sodium concentrations. Sodium concentrations of the water column were not measured in this study and as such no comment can be made upon this aspect of the study. However the 12 hour exposure period is well below the 24 hours at which Lauren and McDonald (1985) observed the disruption to the activity

of Na^+/K^+- ATPase. Grosell and Wood (2002) found the effects upon the apical transport of sodium to occur after 2 hours exposure to copper. It would then appear plausible that in this study the apical transport of sodium has been interrupted causing a depression of tissue levels of sodium whereas the baso-lateral component of sodium transport was not inhibited, and there was no consequent affect upon plasma sodium concentrations.

Aside from these physiological considerations the second set of experiments provides a body of data in well defined conditions cataloguing the gill metal interactions after exposure to waterborne copper and zinc. As noted earlier, mixed metal exposures are an area where data are lacking. Even the simple observations that there was no effect upon the concentrations of zinc, calcium, magnesium and potassium in the gill tissue contributes to the current low level of knowledge in this field of research as well as providing data of competitive interactions of metals at the gill epithelium. While computer modelling was beyond the scope of this study, there is a potential to use these data in future models of metal gill interactions and hopefully contribute further to the scientific investigations in this field.

The third series of experiments, recorded in chapter 4, examined depuration rates of metals from fish gills *post-mortem*. This was necessary to the development of my proposal of metal ratios as biomarkers for fish kills. The decision to include two levels of brackish water broadened the scope of the study and was also pertinent to the involvement of the Department of Primary Industry, Water and the Environment,

Tasmania (DPIWE). Fish farming in Macquarie Harbour has been receiving support from DPIWE since the early 1990s and my involvement provided an opportunity to examine metal ratios in an environmental context and in an area where fish kills had been recorded.

The ratios did not provide definitive evidence of copper induced mortality in the historical fish kills at Macquarie Harbour in south-west Tasmania. Although copper was implicated in the fish kills due to Macquarie Harbour's history of elevated copper concentrations, it is not known that the mortalities were copper induced. Alternatively, the ratios could be influenced by the animal's history of exposure to water of varying quality. However, there is strong evidence these animals were acclimated to copper exposure. As described in chapters 1, 4 and 5, Macquarie Harbour has received copper rich discharge from the Mt Lyell Copper mine for over a century. This has resulted in a copper rich alluvial fan of tailings where the King River discharges into Macquarie Harbour. The harbour consequently has concentrations of copper greater than those normally found in unpolluted water bodies. In the laboratory trials conducted naïve animals were used. Further studies with copper acclimated animals will allow for comparisons between the laboratory data and the fish kill data from areas like Macquarie Harbour. Additionally, the high concentration of zinc found in the gills of the animals from Macquarie Harbour highlighted the need to understand water quality parameters and local conditions.

The two most significant observations from these experiments were firstly, that the metal concentrations of the gill tissue will equilibrate to the ambient concentrations *post-mortem*, and secondly, that animals in isotonic, hypotonic and hypertonic environments will accumulate copper at different rates, although the rate of accumulation is not related to the ionic composition of the ambient environment.

The equilibration of the metal concentrations on the gills to ambient ionic concentrations was more rapid in freshwater than in brackish waters. This was true for all metals examined in the gills and was especially conspicuous for sodium. This observation appears obvious in hindsight but is a necessary piece of information in the broader context of examining fish kills.

That the movement of sodium toward ambient concentrations was particularly pronounced was not surprising given the difference between the cellular sodium levels and those of the ambient environment. These results may be useful to determine the time of the occurrence of fish kills, especially in marine environments. Currently, the time of death is qualitatively assessed by the loss of colour of the gill filaments. The rate of loss or uptake of sodium from the gills will be dependent on the ionic strength of the surrounding waters. In freshwaters this will be variable whereas the water quality of marine environments is more homogenous and the rate of uptake will therefore be more uniform. In freshwaters it would be a simple procedure to run similar experiments to the depuration experiments of chapter 4, in waters of various

hardness and sodium concentrations, to determine the rate of loss of sodium from the gills under different water quality conditions.

The second observation that may warrant further examination is that of the differences in copper accumulation between animals in isotonic, hypotonic and hypertonic environments. As noted in the earlier text, little work has been done on metal toxicity in isotonic environments. The isotonic environment represents the transition from a hypo- to a hypertonic environment and therefore, the transition from the uptake to the extrusion of sodium at the gills of teleosts (Karnaky Jr 1998; Wilson and Taylor 1993b). This is a pronounced alteration of the physiological function of the gills and according to my research also showed a significant alteration in gill/metal interactions at these organs. Further research into these phenomena would certainly be of interest to understanding the functioning of the gills in euryhaline species and the consequences of metal pollution in estuaries.

The potential of metal ratios as biomarkers was again indicated in freshwater trials although the window of opportunity for sampling was less than 6 hours *post-mortem*. Although this is a brief period of time it does provide some sampling opportunity. Metal ratios have an advantage over enzymatic analysis as the metals will not deteriorate, although the possible sampling/collection time is limited by the deterioration of the tissue. Enzyme assays require animals to be still alive during sampling. Thus, these results are a significant contribution to the investigations of mass fish mortalities where copper toxicity was implicated as a causal factor.

The final set of experiments, reported in chapter 5, examined the effects of dissolved organic carbon on copper accumulation at the gills of rainbow trout in brackish waters. This was done in conjunction with measurements of ASV-labile copper to attempt to quantify the bioavailable fraction of the dissolved copper. Waters from Macquarie Harbour were used as this provided important information for the investigation of fish kills and copper toxicity in this fish farming region.

While we could satisfactorily demonstrate the reduction of copper accumulation at the gills as the level DOC increased, the ASV measurements did not correspond well with the reduction in gill binding. In fact, gill binding correlated more strongly with the concentrations of total measured copper. As copper toxicity has been equated with the free ion content of copper in the water column, this suggested ASV copper may not be a good descriptor of the bioavailable fraction of copper in the water column. This finding was in agreement with earlier research by Eriksen et al. (2001) and Stauber et al. (2000).

Limitations of the study

The impetus of this study was to explore the possibilities of finding a biomarker for copper exposure that could be used in remote areas, putatively at mine sites in developing countries. It was also hypothesised that such a biomarker could be utilised by staff at the hypothetical mine site who probably would be technicians or perhaps inorganic chemists, but essentially people without biological training or facilities for

sophisticated biological assays. Therefore the précis of the thesis may be stated as "Can a biomarker for copper exposure be developed using only an atomic absorption spectrometer?" While this may be considered a limitation upon the research conducted, I believe it was also a worthwhile endeavour as, if successful, such a biomarker would be of immediate benefit to the many copper mines located in inaccessible areas and in developing countries.

Although it is not possible to claim unqualified success in the search for such a biomarker, there have been positive developments. Probably the most significant is the potential for Cu/Na ratios to indicate recent acute exposure to copper. As described above, the tissue concentrations of sodium declined within 12 hours of exposure to acute waterborne copper while copper concentrations in the gills increased. Although this is a coarse measurement of gill-metal interactions and is difficult to relate to either the chronic exposures many animals experience through anthropogenic-related copper pollution or normal copper homeostasis, it is precisely such a measurement that is required to satisfy the starting point of this research.

The analyses in this work present the metal binding as the sum of various different compartments and binding sites and may be considered a coarse measurement. The use of gill filaments does not discriminate between intra-cellular and extra-cellular metal. The epithelia have specific binding sites and non-specific binding sites. However, as stated in the précis, the challenge of this research project was to

determine if such a broad determination of metals at the gills could lead to the development of an effective biomarker and I believe some success has been achieved.

Several studies have now been published using Cu^{64} radio-tracers to monitor newly accumulated copper and to differentiate between the different copper pools within teleosts. This has led to some exciting new developments in the understanding of coper metabolism in fish (Grosell 2001; Grosell et al. 1997; Grosell et al. 1998; Taylor et al. 2000; Wood 1992). Again, such subtle and complex experiments were beyond the scope of my research.

Future research needs

Evidence has steadily accumulated in the last decade of the importance of site-specific water quality criteria in determining copper toxicity in freshwater fish. While the copper/metal ratios, and particularly Cu/Na ratios, in the gills have shown some qualified potential as biomarkers of copper exposure, it will be necessary to validate their use in a broad range of conditions. These would include waters of various pH, hardness and DOC levels.

Additionally, the depuration rates of copper from animals exposed to acute but sub-lethal concentration of copper would need to be examined i.e transferring fish that are not moribund from contaminated to clean water. While the rates of clearance of copper may be similar between moribund and 'live' animals, it is not safe to assume this will be so. Recent evidence of copper specific transporters in the gills have

provided strong evidence for active copper transport at the gills of freshwater fish. In the moribund animals, once homeostasis was no longer controlled, all the metals bound with or to the gill tissue started moving toward equilibrium with the environmental levels.

The ratios between copper in the gills and liver also provided some indication of recent acute copper exposure in chapter 2. This is an area that may warrant further attention. Numerous studies have now highlighted the gills as rapidly accumulating copper yet liver copper concentrations require days before being affected by copper exposure. The work focused on the gills and gill/metal interactions so further work on the $gill_{[Cu]}/liver_{[Cu]}$ ratios was not included. Yet even in the second set of experiments (chapter 3) it was apparent the copper concentrations of the gills were elevated within 12 hours while copper levels in the liver were unchanged. Although these analyses were not included in this thesis, it is an area that may yield more useful results in the search for to biomarkers of short-term acute copper exposure.

In conclusion, this thesis has produced a body of data on the subject of short-term, acute copper exposure of rainbow trout covering a variety of exposure conditions including mixed metal exposures, exposure at different salinities and also at different concentrations of DOC in brackish conditions. The initial impetus for the study, to examine Cu/Zn ratios in the gills of fish provided some indications that metal ratios of the gills can be used in this capacity. Further work under mixed metal exposure regimes indicated that Cu/Na ratios may be more sensitive to, and a better indicator

of, copper exposure than other metal ratios in the gills. This observation is due to copper's inhibiting branchial sodium transport such that that gill copper increases and gill sodium decreases. Further studies demonstrated the gill metal ratios may be used *post mortem*, which is advantageous over enzymatic analyses. It was also noted that the sodium concentrations of the gills may be able to quantify the time since death in fish kills. The experiments in brackish waters recorded different accumulation rates of copper in the gills of animals in hypo, iso and hypertonic environments. An 'ion exchange' model of copper elution at the gills was proposed to account for these differences. It was also considered as an indication that the changing physiological function of species in waters of different ionic strengths may be an important consideration for studies of copper toxicity in these different environments. Finally, the effect of DOC on copper's bioavailability and consequent binding to the gill epithelium of rainbow trout was examined in brackish water. While DOC reduced the binding of copper to the gills, it was found that ASV-labile copper did not accurately assess the bioavailable fraction of the copper in the water column.

These findings will contribute both to the database of knowledge concerning copper exposure, particularly in the field of mixed metal exposure where more data is needed and also in brackish waters. The use of metal ratios in the gills as biomarkers deserves further attention, particularly the Cu/Na ratio as it fulfils the criteria stated at the beginning of this thesis. It is a simple method which can be used routinely with equipment available at mine sites and in remote areas by people without biological training. It is also specific to copper exposure. Therefore, although further work and

refinement will be necessary, this work provides a basis to pursue such a biomarker and to provide a tool for the analysis of both fish kills and fish exposed to short-term acute copper exposure.

It is the recommendation of the author that in the event of a fish kill scenario the following procedures should be adopted by investigators for the sampling and analysis of the gills for metal concentrations.

1. The gills be immediately excised from the animal and placed in zip-lock sample bags before being placed on ice. The sample number, date, location and type of fish should be recorded both with the sample and on a record sheet, along with any relevant comments. The gills should be frozen at - 20° C as soon as possible.

2. Filaments are to be dissected from the arches prior to digestion. Between 50 – 200 g of filaments are required. In animals of 200 g and above the filaments of a single arch will normally provide sufficient material for the digestion process. For animals of lesser weights it may be necessary to remove the filaments from more arches.

3. Digest the filaments in 5 mL of concentrated nitric acid in a microwave digester. After digestion the solute should be transferred to a 10 mL volumetric flask and made up to volume using deionised water. Blanks of

deionised water must be included with each batch of digestions. In analytical labs were many different analytical processes are conducted it is important to wash benches and equipment, including all glassware, with dilute nitric acid (5 – 10%) followed by rinsing with deionised water to prevent contamination of samples occurring.

4. Analysis of the various metals of interest may be conducted using flame atomic absorption spectrometry. Copper, zinc, and magnesium were analysed in this thesis using an acetylene/air flame while calcium was analysed using a nitrogen oxide/air flame. Sodium analyses were conducted by emission spectroscopy. Standard methods for the operations of AAS are described by manufacturers, including the preparation of standard curves.

5. For some metals it may be necessary to dilute the samples to match the range of the standard curve. A series of preliminary dilutions with 3 – 5 samples at 1:10, 1:50, 1:100, 1:500 and 1:1000 should indicate which dilution is most appropriate for a specific analysis.

6. Where possible, animals from unaffected waterways within the same system should be sampled following the same procedures to provide baseline data of the metal ratios in the gills of fish.

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