ENVIRONMENTAL CHEMISTRY, PHYSIOLOGICAL HOMEOSTASIS, TOXICOLOGY, AND ENVIRONMENTAL REGULATION OF COPPER, AN ESSENTIAL ELEMENT IN FRESHWATER FISH

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ABSTRACT

Exploitation of natural mineral deposits and subsequent re-deposition in aquatic environments at levels potentially harmful to biota necessitate regulation of environmental discharge of metals. Proper environmental regulation of metals requires adequate knowledge on the bioavailablility and effects of metals on aquatic organisms. Moreover, the need to better understand biological effects of metals has given impetus for studies on the homeostatic physiology of metals because the root cause of toxicity is the breakdown of homeostasis. In this paper we review recent advances in the understanding of the environmental chemistry, toxicology, and physiological homeostasis of copper (Cu) in freshwater systems. Present water quality criteria do not adequately consider bioavailability and metal homeostasis. We show that consideration and incorporation of recent knowledge on bioavailability, homeostatic physiology, and acute and chronic toxic effects of Cu greatly improve the predictive precision of models, such as the Biotic Ligand Model, for ecological risk assessment and environmental regulation of Cu, and reduces the need for reliance on the Precautionary Principle. Furthermore, we highlight present gaps in knowledge of environmental physiology, homeostasis, and toxicology of Cu and suggest directions for future research.

Key words: Copper, environmental chemistry, homeostatic physiology, toxicology, environmental regulation, BLM, fish.

INTRODUCTION

Human activities such as mining have increased global fluxes of trace elements such as Cu to aquatic systems, resulting in negative impact on aquatic organisms and a need to regulate metal discharge into the environment. Although good progress has been made in our understanding of toxicology, environmental chemistry, physiology, bioavailability, and pathways of Cu uptake (Wood 2001; Clearwater et al. 2002; Gorsuch et al. 2002), these advances have not been adequately integrated in the approaches used in setting water quality criteria (WQC) and ecological risk assessment (ERA). Copper occurs ubiquitously in natural waters either in a free ionic (aq) form, complexed by organic and inorganic ligands or associated with suspended particulate matter (Achterberg et al. 2002). The free Cu2+ ions and the CuOH+ species appear to be the most bioavailable and toxic forms of Cu (Allen and Hansen 1996). With the exception of lipid-soluble complexes, organically complexed waterborne Cu is generally unavailable and non-toxic (Campbell 1995; Allen and Hansen 1996; Croot et al. 1999). Thus it is important to consider speciation and Cu bioavailability when setting WQC. Indeed, it has been recognised that current approaches do not assess the true ecological impact because they are based on total or dissolved metal and ignore dietary exposure (Bergman and Dorward-King 1997). Moreover, the potential for toxicity as well as deficiency underpins the need to understand the physiological homeostasis of Cu in the development of WQC for Cu because likelihood for deleterious effects just if regulations are written above or below the optimal values. Earlier studies investigated waterborne and dietary Cu exposures independently, and mainly from a toxicological perspective (Handy 1996; McDonald and Wood 1993). While toxicological studies have continued to increase our knowledge on Cu toxicology (Wood 2001; Clearwater et al. 2002), several recent studies have examined Cu homeostasis and have demonstrated interactions between waterborne and dietary pathways of uptake (Grosell et al. 1997, 1998a,b, 2001b; Clearwater et al. 2000; Kamunde et al. 2001, 2002a,b, 2003; Bury et al. 2003; Pyle et al. 2003) and environmental chemistry (Achterberg et al. 2002; Bazzi et al. 2002; Lu and Allen 2002). An opportunity exists to improve the approaches used for deriving WQC and ERA of Cu by consolidating and synthesizing these data. In this review therefore, recent advances in environmental chemistry, toxicology, environmental regulation, and physiological homeostasis of Cu are summarised. We emphasise the need to understand the homeostatic physiology, chronic effects, and bioavailability of Cu to set realistic water quality standards that are protective to aquatic biota, yet not unduly detrimental to the metals industry. Integration of the information summarised herein into approaches for WQC and ERA, and the performance of targeted research to fill in specific knowledge gaps identified herein will greatly minimise the application of the Precautionary Principle in environmental regulation of Cu. Moreover, many of the issues addressed here are not unique for Cu, but rather apply generally for other metals.

Environmental chemistry of copper in freshwater

Concentrations of Cu in freshwater environments range from <1 to 9000 µg Cu/L (Leland and Kuwabara 1985) depending on level of natural and anthropogenic inputs. In most unpolluted aquatic habitats concentrations (range <1-6 µg/L) are below the threshold for detectable effects (10-1000 µg/L) on fish (Spear and Pierce 1979; Wood 2001). Elevated levels of Cu in the environment are not necessarily undesirable because Cu is essential for normal growth of fish and is also commonly used to purify and distribute drinking water and to combat the growth of unwanted organisms that foul water intake lines and aquaculture facilities. Therefore for toxicology the issue is determining what levels of Cu, under site-specific conditions, present a risk to fish and other aquatic organisms. Moreover, accumulation and effects do not depend on the total concentrations in the water but rather on the bioavailable fraction (Campbell 1995), which in turn is governed by the overall physico-
Environmental regulation of copper

In aquatic toxicity studies, speciation measurements are obligatory to determine the fraction of total dissolved metal (the ‘toxic fraction’) that will bind to or be transported across a biological membrane such as a fish gill. Speciation models, eg. MINEQL+ (Schecher and McAvoy 2001) and MINTEQA2 (Allison et al. 1991) allow the determination of the metal species in dissolved phase for better characterization of toxic threshold. Generally Cu\(^{2+}\) predominates in waters of low pH, but as the pH increases the contribution of Cu\(^{2+}\) to total Cu decreases with simultaneous increase in the contribution of CuCO\(_3\) and CuOH\(^+\). At typical pHs of natural freshwaters, CuCO\(_3\) is the dominant species while hydroxides and ionic Cu\(^{2+}\) contribute relatively minor proportions (Figure 1). The free form (Cu\(^{2+}\)) and the hydroxide, rather than the carbonate, seem to be the major contributors to acute toxicity (Allen and Hansen 1996).

Although the environmental chemistry of Cu is extensively studied in marine and estuarine waters, fewer investigations have focused on the freshwater environment (Allen and Hansen 1996; Antello et al. 1998; Gardner et al. 2000; Bazzi et al. 2002). A unifying theme of these studies is that Cu binds with ligands present in the water such as natural organic matter, carbonates and hydroxides, rendering it less available for uptake by aquatic organisms. Moreover, it has been shown that organically complexed forms of Cu are substantially less toxic than the free metal ion or inorganically associated species. This is because complexes of Cu with ligands of high binding capacities (eg. dissolved organic matter [DOM]) generally have high conditional stability constants (log K values). Therefore they are less bioavailable, and less toxic to aquatic animals (Allen et al. 1980; Allen and Hansen 1996; Erickson et al. 1996). In natural waters, only a very small percentage of the dissolved Cu is present as free aquo ion (Cu\(^{2+}\)); most of the Cu is adsorbed to colloidal particles or combined in complexes. For example, a recent study in lake Michigan (Bazzi et al. 2002) reported that the total dissolved Cu existed predominantly (more than 98%) as stable, largely non-bioavailable organic complexes. Similar observations have been made in metals contaminated lakes in the Rouyn-Noranda area in Ontario, Canada (Campbell et al. 2003). The exact nature of the metal-complexing ligands in natural waters varies with location but they are thought to include humic substances, synthetic compounds like EDTA, bacterial and algal cells and their breakdown products, and sulfides (Xue et al. 1995; Achterberg et al. 2002).

The Cu-binding affinity and the relative abundance of these ligands appear to be the main factors governing the complexing capacity of natural waters. Thus it is not uncommon to find natural waters containing low concentrations of DOM complexing more Cu than waters with much higher DOM levels (Achterberg et al. 2002), possibly because other ligands are more abundant or have greater Cu binding capacity than DOM. Recently Lu and Allen (2002) reported that Cu binding by DOM from three freshwater sources was similar and the small differences in binding affinities could be partly explained by the humic acid/fulvic acid ratio. By alkalimetric titration the authors described two Cu binding sites on DOM, phenolic and carboxylic sites, and demonstrated that under natural

![Figure 1: Typical distribution of inorganic Cu species within the pH range of natural freshwaters (adapted from De Schamphelaere et al. 2002).](image-url)
water conditions the majority of the Cu-DOM complexation occurs at the phenolic sites, and that the complexation was due to displacement of H\(^+\) by Cu\(^{2+}\) at these sites. Moreover, the phenolic (high affinity) sites are a small proportion of the total sites and major water cations like Ca and Mg do not comprehensively compete with Cu for DOM because they bind mainly to the carboxylic sites.

Most of the approaches currently in use for derivation of WQC and ERA for Cu now recognise the importance of bioavailability (EC 1993; CCME 1999; MacRae et al. 1999; ANZECC and ARMCANZ 2000; USEPA 2001; Meyer et al. 2002) but regrettably consider only water hardness and dissolved metal. Furthermore, recent developments now recognise the importance of considering not only the physico-chemical factors affecting bioavailability, but also the biotic component, in WQC and ERA approaches. The process of integrating the biotic and abiotic components into a mechanistic bioavailability-based tool for deriving WQC and ERA for metals is referred to as the Biotic Ligand Model (BLM, Di Toro et al. 2001, Santore et al. 2001, Paquin et al. 2002) and will be discussed in a later subsection in this review. For now it will suffice to say that the dominant bioavailable and toxic Cu species in freshwaters are Cu\(^{2+}\) and CuOH\(^+\), although CuCO\(_3\) (aq) and [Cu(OH)]\(^{2+}\) have also been reported to be toxic (Magnuson et al. 1979, De Schamphelaere et al. 2002). The relative proportions of these Cu species depend on the ambient physico-chemical characteristic such as pH (see Figure 1). The affinity for the biotic ligand is in the order Cu\(^{2+}\) > CuCO\(_3\) (aq) > CuOH\(^+\) for Daphnia (De Schamphelaere et al. 2002) and it is likely that the same applies in fish. While considering Cu speciation, it is important to recognise that speciation events in the gill microenvironment may be different from those in the surrounding water and the fish microenvironment, due to the pH changes in the vicinity of gills occasioned by excretion of H\(^+\), CO\(_2\), HCO\(_3\) and NH\(_3\) (Playle and Wood 1989; Randall et al. 1991; Playle et al. 1992; Tao et al. 2000). For waterborne Cu toxicity, speciation events in the gill vicinity are important because gills are the primary targets for the acute toxic effects of Cu (McDonald and Wood 1993; Wood 2001).

Physiology of copper in freshwater fish

Copper, unlike many other potentially toxic metals, is required in trace amounts for normal physiology of all organisms including freshwater fish. It is a cofactor of a number of proteins including superoxide dismutase, caeruloplasmin, and cytochrome c oxidase that are essential for key biochemical functions. The biochemical function of Cu is vested in its ability to cycle between two redox states (Cu(I) and Cu(II)) but this very property is also the basis for its toxicity. Although it is agreed that the maintenance of Cu levels adequate to meet physiological needs is a necessity for all living organisms, the mechanisms by which fish achieve Cu homeostasis are not well understood at present. It is generally believed that to meet the physiological requirement, fish need to consume dietary Cu at a concentration of 3-35 µg/g depending on species (Clearwater et al. 2002). However, recently Kamunde et al. (2002b) showed that by considering the input from water when determining Cu requirement in fish, as little as 0.8 µg/g Cu in the diet is adequate to support normal growth in rainbow trout juveniles (normal) water Cu level of 3 µg/L. The same level of dietary Cu is inadequate at less than 1µg/L, waterborne Cu (Kamunde et al. 2002b). Although not well characterised, fish are presumed to possess a tightly regulated homeostatic mechanism responsible for the uptake and distribution of Cu to ensure that toxicity or deficiency do not occur. Most commonly, breakdown in Cu homeostasis manifests as toxicity both in laboratory and in nature; deficiency is rare and has only been demonstrated in laboratory experiments in catfish (Murai et al. 1981), Atlantic salmon fry (Berntssen et al. 1999), and rainbow trout juveniles (Kamunde et al. 2002b). However, this does not necessarily mean that Cu deficiency in natural populations of fish does not exist. It is quite possible that in waters with low levels of Cu and high concentrations of ligands of high complexing capacities, Cu can be rendered unavailable to the extent that uptake is inadequate.

There does not seem to be a constant whole body Cu concentration in fish; Cu concentration changes as fish grow (Shearer 1984). For example in rainbow trout, whole body Cu concentration in healthy Cu-unexposed individuals ranges between 0.6 and 1.5 µg/g Cu (Shearer 1984; Laurén and McDonald 1987a; Hardy 1992; Marr et al. 1996, Kamunde et al. 2001, 2002a,b) but this value can increase 6—10-fold with (Mount et al. 1994; Marr et al. 1996) and without (Kamunde et al. 2001, 2002b) adverse effects. However, a decline in concentration to approximately half of the basal concentration in previously unexposed juvenile rainbow trout resulted in deficiency (Kamunde et al. 2002b). Thus it appears that fish have a greater safety window for withstanding increases than decreases in whole body Cu concentration. The mechanisms responsible for the ability of fish to withstand high increases in Cu concentrations are discussed below (see Acclimation).

Complex organisms such as fish achieve Cu homeostasis not only at the cellular level but also at the tissue and organismal levels. Generally Cu homeostasis entails regulated uptake, distribution, and excretion, and occurs by coordinated interactions of several organ systems (Figure 2). In the ensuing section we discuss recent salient advances in our understanding of each of these phases of Cu homeostasis.

**Copper uptake**

**Copper uptake from water**

Uptake of Cu from water has been extensively studied particularly as it relates to toxicology (McDonald and Wood 1993; Wood 2001). Because Cu is an essential metal, background concentrations in tissues are relatively high compared to non-essential metals. A consequence of the high background Cu levels coupled with the fact that Cu is homeostatically regulated is that it is impractical to use exposures to non-radioactive Cu to assess uptake rates. Measurement sensitivity is inadequate. Moreover, exposure concentrations would have to be relatively high to attain measurable uptake, thus posing a potential to injure cells, such that the measured rate might not be physiological. To this end, radioisotope methodology (Wood 1992) using ⁶⁵Cu (t\(_{1/2}\) = 12.7 h) has been utilised in several studies to determined realistic rates of Cu uptake from water (Laurén and McDonald 1987a; Kamunde et al. 2001, 2002a,b, 2003; Kamunde and Wood 2003).

Several factors including water chemistry and body size influence the rate of Cu uptake in freshwater fish (Table 1). Kamunde et al. (2001) described exponential decline in whole body Cu uptake rate with increasing body mass in trout, and more recently Taylor et al. (2002) demonstrated that uptake into the gills also declines exponentially with body mass. The size-dependent uptake probably
explains, at least in part, the commonly observed higher sensitivity of small (young) fish to Cu (Spear and Pierce 1979). As well, rates of Cu uptake from water appear to be influenced by the body Cu content. A decline in whole body Cu content, for example in experimentally-induced deficiency, increased waterborne Cu uptake rates, whereas an increase in whole body Cu content from previous exposure to either elevated dietary or elevated waterborne Cu decreased Cu uptake (Kamunde et al. 2001; 2002a,b). Clearly, waterborne Cu uptake by the gills is homeostatically regulated. Recently we observed that fish maintained on high rations had higher rates of waterborne Cu uptake relative to fish on low rations, possibly to meet increased demand of Cu for rapid growth (Kamunde and Wood 2003). Modulation of dietary quality, specifically exposure of fish to elevated dietary Na, was also recently reported to decrease the uptake rate of both waterborne Cu and Na (Kamunde et al. 2003; Pyle et al. 2003). Furthermore, these authors demonstrated a strong correlation between Cu and Na uptake during waterborne radiotracer exposures to $^{64}$Cu and $^{22}$Na in which >95% of the Cu uptake was explained by the Na uptake, which strongly suggested that Cu and Na share uptake pathways, at least partially. Note, however, that in absolute terms the uptake rate of Na is almost 4 orders of magnitude higher than Cu uptake; for every mole of Cu taken up from the water, nearly 6000 moles of Na are taken up (Kamunde et al. 2003).

The mechanisms of waterborne Cu uptake in fish have been the focus of recent research (Wood 2001; Grosell and Wood 2002; Handy et al. 2002; Kamunde et al. 2002a, 2003; Pyle et al. 2003) and although much remains to be done, several aspects of Cu uptake via the gills have now been characterised. In Figure 3 we summarise our current understanding of Cu uptake mechanisms in fish gills. Available evidence points to at least two pathways of Cu uptake at the fish gills, namely Cu-specific and Na-linked pathways. The best characterised are the Na-linked pathways. It was recognised more than 30 years ago that exposure to elevated waterborne Cu reduced plasma Na and Cl levels (McKim et al. 1970; Lewis and Lewis 1971). Later Laurén and McDonald (1985, 1986) demonstrated that this was due to disruption of gill ionoregulatory

Figure 2. Schematic diagram of whole body Cu metabolism in fish. Whole body Cu metabolism involves coordinated interactions of several organ systems. The gut and the gills are the principal sites of Cu uptake and at typical background concentrations of Cu, gastrointestinal absorption accounts for the majority of Cu uptake. Copper is transported via blood plasma in the primary phase of transport to the liver, which occupies a central locus in Cu metabolism. In the liver Cu is incorporated into various proteins for biological function, detoxification, and storage. Protein bound Cu (primarily caeruloplasmin-bound Cu) enters the secondary phase of transport to the rest of the body. Copper uptake is balanced by excretory losses via bile, the gut, and gills, and other less important losses via the kidney and the rest of the body (miscellaneous losses).
function. Sodium uptake in freshwater fish probably occurs via the proton-pump-powered epithelial Na channel (ENaC) with subsequent export into blood via the basolateral Na,K-ATPase (Lin and Randall 1995; Sullivan et al. 1995; Bury and Wood 1999; Wood 2001). Evidence that Cu enters the gill cells via the ENaC has been provided directly by Na-competition experiments and pharmacological studies (Grosell and Wood 2002), and indirectly by manipulation of the activity of Na uptake pathways with dietary Na (Kamunde et al. 2003; Pyle et al. 2003). In these respects, it appears to be similar to silver (Bury and Wood 1999). That Cu enters via the Na channel in competition for Na, despite very different chemistries of these two elements, has been explained on the basis that the hydrated molecules of Cu and Na have very similar mobilities (Handy et al. 2002).

The Cu-specific pathway is insensitive to Na-competition or to pharmacological blockade of ENaC (Grosell and Wood 2002). This pathway is much less well characterised but apical entry is likely via an apical high affinity Cu transporter (CTr) analogous to hCTr1 (Zhou and Gitschier 1997; Lee et al. 2000, 2001, 2002; Harris 2001). An analogue of hCTr1 has recently been cloned from zebrafish embryos (Mackenzie et al. 2004) and while the precise location is not known at present, the gill is a prime candidate. For both apical uptake pathways, the basolateral export is likely through a Cu-ATPase although we cannot at present eliminate the possibility that low levels of Cu below the threshold needed to inhibit basolateral Na,K-ATPase (Li et al. 1996, 1998) may in fact be transported by this enzyme. Although not conclusive, vanadate (P-type ATPase blocker) sensitivity of branchial Cu uptake (Campbell et al. 1999) and partial cloning of a Cu-ATPase (Grosell et al. 2001a) are strong pointers to the existence of a Cu-ATPase in the fish gills.

The specific Cu species that traverses the branchial epithelium has not been fully characterised. However, one popular theory is that for both uptake pathways, Cu$^{2+}$ may be reduced to Cu$^{+}$ by reductases on the gill surface before uptake (Grosell et al. 2002; Handy et al. 2002), because studies in other systems indicate that Cu-ATPase and hCTr1 transport Cu$^{+}$, and not Cu$^{2+}$ (Harris 2001; Lee et al. 2002). Additional support for this theory comes from the demonstration that in lamprey erythrocytes Cu$^{2+}$ ions are reduced to Cu$^{+}$ ions on the membrane surface prior to absorption (Bogdanova et al. 1999).

Copper uptake from food

Reinfelder et al. (1998) argue that bioavailability of metals in the gut may not depend on free ion concentration since all metal in the gastrointestinal tract is probably bound to biological material which is subsequently absorbed. However, bioavailability and assimilation efficiency of dietary Cu are affected by several factors including diet quantity and quality, and the concentration of Cu in diet. For example, assimilation/retention efficiency of Cu decreases with dietary concentration or dose (Handy 1996; Clearwater et al. 2000, 2002; Kamunde et al. 2001, 2002b; Kamunde and Wood 2003) indicating that uptake mechanisms saturate at higher levels of Cu, and that Cu transport mechanisms exist in the gut. Thus concentration-dependent change in assimilation efficiency is a potential mechanism for regulating uptake and accumulation of dietary Cu in fish.
To the best of our knowledge, only one study (Kamunde et al. 2002a) has directly measured the rate of Cu uptake from food. In this study, fish maintained at 14°C and voluntarily fed a control diet spiked with 64Cu took up Cu at a rate of 0.9 ng/g/h, ten times higher than the rate of waterborne Cu uptake determined in control hard water during the same study. Gut tissues saturated in concentration within 12-24 h and the posterior intestine was the main site for Cu uptake. Other recent studies have assessed the mechanisms of Cu uptake using gut sacs (Handy et al. 2000) and cannulation of the stomach in intact fish (Clearwater et al. 2000) and provide most of the current knowledge on mechanisms of gastrointestinal Cu uptake in fish. Evidence from these studies suggests that the gastrointestinal apical Cu entry is passive while the basolateral export is the rate-limiting step in intestinal uptake of Cu. Handy et al. (2000) described concentration dependent changes in basolateral Cu absorption across the catfish gut and postulated the presence of a Cu-ATPase and a Cu/anion symport, while Q10 analysis (Clearwater et al. 2000) suggested that apical entry was by diffusion and basolateral exit was biologically mediated. In mammalian intestine, an uptake phase independent of the ATP status of the cell and an energy dependent rate-limiting transfer step has been described (Linder and Hazegh-Azam 1996). A role for the major plasma membrane Cu transporter identified so far, the high affinity Cu transport protein (Ctr1), in the apical Cu uptake has been demonstrated (Zhou and Gitschier 1997; Lee et al. 2000, 2001, 2002; Harris 2001). In addition, the natural resistance-associated macrophage protein (Nramp2) system (also called DMT1 or DCT1), the main iron transporter in intestinal cells, is thought to mediate uptake of Cu through an energy independent mechanism (Rolfs and Hediger 1999). These mechanisms of Cu transport likely exist in fish gut as well because Nramp is present in fish genome (Dorschner and Phillips 1999; Donovan et al. 2002) and a high affinity Cu transporter homologous to the hCtr1 has recently cloned in zebrafish embryos (Mackenzie et al. 2004).

Figure 3. Schematic diagram of a branchial cell showing the pathways of Cu uptake and the sites of toxic action in freshwater fish. The mechanisms Cu uptake and toxicity are intricately linked to branchial Na+ transport pathways. Apical Cu entry occurs via Cu-specific pathways (Ctr) and by competition with Na+ via the ENaC or the Na+/H+ exchanger, while basolateral exit occurs through a putative Cu-ATPase and/or Na+/K+-ATPase. The toxic action is predominantly via impairment of Na+/K+-ATPase, ENaC and destruction of cell junctions, and possibly also via poisoning of the Na+/H+ exchanger and carbonic anhydrase. * Indicates that mechanism is yet to be fully characterised.
It therefore does appear that there are major similarities between mammalian and fish intestinal Cu transport but more research is required to bridge the gaps in knowledge.

**Interactions between waterborne and dietary Cu uptake**

Until recently the relative importance of water and diet as sources of Cu for fish was unknown. Recent studies investigating interactions between branchial and gastrointestinal uptake of Cu (Kamunde et al. 2001, 2002a,b) have demonstrated that under normal levels of Cu in the water and food, rainbow trout derives over 80% of its Cu requirement from food. Moreover, the rate of internalisation of dietary Cu is more that ten times greater than that of waterborne Cu. The relative contribution of waterborne uptake is variable and becomes greater at low levels of Cu in the food and at high levels of Cu in the water. Thus under experimentally induced deficiency, up to 60% of body Cu burden was attributed to waterborne uptake (Kamunde et al. 2002b), while at 20 µg/L waterborne Cu, almost 50% of the body Cu burden was attributable to waterborne uptake (Kamunde et al. 2002a). An earlier study (Miller et al. 1993) also found that the relative contribution of Cu to Cu accumulation in the liver increased as waterborne Cu level increased. Interestingly, Zn, another essential trace element, behaves in a similar fashion (Spry et al. 1988). Clearly, although it is commonly assumed that “normal” Cu uptake occurs almost entirely from the diet, and that the entry of Cu (and other metals like Zn) across the gills is “pathological”, branchial Cu uptake plays an important role in the normal metabolism of Cu in fish. A recent review on the nutritive uptake of metals (Bury et al. 2003) further highlights the importance of branchial uptake in normal metabolism of essential metals. The role of the gill in nutrition of essential metals including Na+, Cl-, and Ca2+ is even greater, with time and do not appear to saturate during long-term dietary exposure to waterborne Cu (Kamunde et al. 2001, 2002b), while at 0.6 – 0.8 µg/mL (Grosell et al. 1997, 1998b, 2001b; Kamunde et al. 2001). It appears that Cu is rapidly cleared from plasma such that as much Cu as enters leaves almost immediately; a linear increase in 64Cu radiolabel in plasma over time does not translate into increased total plasma Cu concentration (Grosell et al. 2001b; Kamunde and Wood, unpublished). Muscle Cu concentrations remain low during both dietary and waterborne exposures at concentrations similar to those in blood plasma (Grosell et al. 1996; Kamunde et al. 2001; Clearwater et al. 2000, 2002). However, Cu in muscle can contribute a substantial proportion of the body Cu burden because of the relatively high muscle mass in fish.

**Excretion of copper**

Several recent studies indicate that exposure to elevated Cu via water and/or via the diet stimulates Cu excretion into bile (Andreasson and Dave 1995; Grosell et al. 1996, 1997, 1998a,b, 2001b; Kamunde et al. 2001; Clearwater et al. 2000, 2002). A small fraction of the Cu transported to the kidney may be excreted through urine, although renal excretion appears to contribute minimally to total excretory losses of Cu (Grosell et al. 1999b). The gastrointestinal tract, in addition to providing a conduit for excretion of biliary Cu, may participate directly in Cu excretion via intestinal fluids or exfoliation of Cu-impacted intestinal epithelial cells. Several authors have reported increased apoptosis (Bemtssen et al. 1999; Kamunde et al. 2001) during exposure to elevated levels of dietary Cu. Moreover, induction of metallothionein in gut tissue (Handy et al. 1999) may facilitate excretion by sequestering the Cu in intestinal tissue, with elimination occurring through epithelial cell sloughing. Another potential Cu excretion pathway is via the gill. Assessment of extra hepato-epibiliary Cu excretion, likely through the gills, revealed that Cu loss through this pathway substantially exceeded loss through the bile (Grosell et al. 2001b). Although

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Environmental regulation of copper

Kamunde and Wood

Indeed Cu accumulation in the gut is diagnostic of dietary exposure since accumulation of waterborne Cu in the gut is minimal (Kamunde et al. 2001, 2002a). In the long-term, the liver is the main site of Cu accumulation regardless of the route of exposure (Laurén and McDonald 1987a; Grosell et al. 1996; Clearwater 2000, 2002; McGeer et al. 2000b; Kamunde et al. 2001, 2002a,b), presumably due to high levels of metal binding proteins in this tissue. Copper from dietary uptake is channelled directly to the liver via the portal circulation leading to greater hepatic accumulation than during waterborne exposure since Cu from branchial uptake may accumulate in other organs before reaching the liver. This apparent significant first-pass effect of dietary Cu may partly explain its lower toxicity relative to branchial Cu exposure because other organs are protected. In the liver, Cu may follow one of three fates as is the case in mammals: i) incorporation into transport proteins such as ceruloplasmin for secondary transport into other tissues ii) incorporation into cupro-proteins, eg. metallothionein for biological function or storage or iii) excretion via bile. Whole body Cu levels vary depending on the prevailing waterborne and dietary Cu levels, body requirements, and growth rates.

Copper also accumulates in the kidney irrespective of route of exposure (McGeer et al. 2000b; Kamunde et al. 2001) although concentrations attained are much lower than those in the liver. Interestingly, plasma Cu levels appear to be maintained constant over a wide range of sublethal waterborne and dietary Cu exposure levels; for rainbow trout, plasma Cu levels appear to be regulated at 0.6 – 0.8 µg/mL (Grosell et al. 1997, 1998b, 2001b; Kamunde et al. 2001). It appears that Cu is rapidly cleared from plasma such that as much Cu as enters leaves almost immediately; a linear increase in 64Cu radiolabel in plasma over time does not translate into increased total plasma Cu concentration (Grosell et al. 2001b; Kamunde and Wood, unpublished). Muscle Cu concentrations remain low during both dietary and waterborne exposures at concentrations similar to those in blood plasma (Grosell et al. 1996; Kamunde et al. 2001; Clearwater et al. 2000, 2002). However, Cu in muscle can contribute a substantial proportion of the body Cu burden because of the relatively high muscle mass in fish.

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AUSTRALASIAN JOURNAL OF ECOTOXICOLOGY Vol. 10, pp. 1-20, 2004
specific studies are required to determine the real significance of this pathway of Cu excretion, accumulation of Cu at the gill following dietary exposures (Handy 1996, Kamunde et al. 2001, 2002a,b) strongly supports a role of the gill in Cu excretion. Copper excretion need to be studied in detail to gain insights on the kinetics of Cu loss in fish and the relative contribution of all potential excretory pathways. In mammals, which have one major route of excretion, the rate of loss is exponential and biphasic, with a half-life of 67 h for the first 72 h followed by a much slower loss rate in subsequent days (Barceloux 1999). Kinetics of Cu loss in fish are likely to be more complex due to significant multiple pathways of excretion.

**Copper homeostatic system in fish**

Based on the information discussed above, we speculate that there is a whole-body Cu homeostatic system that maintains constant plasma Cu concentration (Figure 4). This system may encompass central sensors, possibly within the central nervous system or blood circulatory system, and local sensors within target tissue cells. Moreover, because of a demonstrated linkage between Na⁺ and Cu metabolism (Handy et al. 2002; Grosell and Wood 2002; Kamunde et al. 2003; Pyle et al. 2003), a partial linkage with the Na⁺ sensing mechanisms cannot be ruled out. In this homeostatic system, information (possibly neural and/or hormonal) on changes in plasma Cu levels is relayed to the regulatory organs, which for fish are probably the gills, gut, and the liver. Depending on the nature of the change in plasma Cu concentration, increased or decreased uptake occurs at the gill and gut concurrent with increased or decreased hepatobiliary excretion. Cu translocating proteins, such as the Cu-ATPases, within these organs likely mediate these changes. The net effect is the return of plasma Cu to normal levels, which are 0.6 - 0.8 μg/mL in rainbow trout.

The goal of maintaining constant plasma (extracellular) Cu concentration is to satisfy the changing Cu requirements of the cells. Although the mechanisms of Cu regulation in vertebrate cells have not been fully defined, physiologically essential levels of cellular Cu can be achieved via a coordinated series of interactions between transport proteins, vesicles, and soluble peptides (Schaefer and Gatlin 1999; Harris 2001). These transport molecules are part of a Cu homeostatic system that regulates cellular Cu uptake, transport, storage, and efflux. Several aspects of cellular Cu homeostasis in fish remain unclear. For example the specific proteins involved, the regulatory endpoint, the sensing mechanisms and their location, and the mode of integration of the system remain to be decisively determined. Most of the studies addressing cellular Cu homeostasis have been carried out using simple model systems like yeast, and thus do not address the complexity of higher organisms. In yeast, regulator proteins act as Cu sensors that trigger the shutdown of uptake proteins when intracellular Cu levels increase (Harris 2001). Alternatively, vesicles with Cu-transporting ATPases work in conjunction with mobile Cu carriers (e.g. chaperones) to get rid of excess cytoplasmic Cu or activate enzymes such as Cu/Zn-superoxide dismutase that protect the cell from Cu-mediated oxidative damage.

Levels at which cellular Cu is maintained are unknown. However, due to a great variability in Cu concentrations in various tissues, cell-type specific Cu-levels are likely. This raises the speculation that possibly only Cu levels within some sensitive cell compartment is regulated. To this end, free cytosolic Cu is low (O’Halloran and Culotta 2000) and it is therefore likely that Cu homeostasis is geared toward satisfying cellular Cu requirements while maintaining safe levels of cytosolic freely dissociable Cu. In vertebrates, no system for sensing Cu has yet been identified (Harris 2001) although metal binding sites on the Cu transporting P-type proteins have been proposed as potential sensors of cellular Cu levels (Strausak et al. 1999). Evidence for this comes from the observation that metal binding sites at the N-terminus of Cu-ATPase bind Cu(I) with high affinity and are involved in ligand exchange with Cu chaperones. Clearly future research on Cu homeostasis in fish needs to characterise the regulatory endpoint (both at organismal and cellular levels) and identify the nature and location of the sensing mechanisms, and the mode of integration of the various components of the system. Moreover, the Na-Cu linkage needs to be explored in depth to fully understand the relative contributions of Na-linked and Cu-specific pathways in Cu metabolism. Such future research would not only illuminate Cu homeostasis in fish, but also lead to a better understanding of both acute and chronic toxicity, and improvement of the environmental regulatory approaches for Cu.

**Toxicology of copper**

Copper toxicity results when the homeostatic mechanisms described above fail, due to acute or chronic exposure to elevated levels of Cu. Fundamentally, the redox cycling of Cu, which is the basis for its biological function, is also partly the basis for the toxicity. Redox cycling of Cu leads to the formation of highly reactive oxygen species (Halliwell and Gutteridge 1984), which can cause devastating cellular damage including oxidation of proteins, membrane lipid peroxidation, and cleavage of DNA and RNA molecules. In addition, Cu can interfere with the homeostasis of other elements (e.g. Na and Zn) and inactivate proteins by strongly binding to cysteine, histidine, and methionine residues. In the following sections we highlight recent developments in the understanding of toxicity of Cu in freshwater fish.

**Acute toxicity of waterborne copper in freshwater fish**

Acute toxicity of waterborne Cu in fish is a well-studied subject that has been periodically reviewed over the years (Spear and Pierce 1979; Flemming and Trewors 1989; McDonald and Wood 1993; Taylor et al. 1996; Wood 2001). In addition, acute waterborne Cu toxicity is well correlated with gill total Cu burden (MacRae et al. 1999). Generally the levels of waterborne Cu that induce acute toxicity in freshwater fish depend on several abiotic and biotic factors (Table 1). Water chemistry parameters, eg. hardness (Pagenkopf 1983; Erickson et al. 1996, 1997; Welsh et al. 2000), alkalinity (Chakoumakos et al. 1996, 1997; Welsh et al. 1999; McGeer et al. 1996; Hollis et al. 1997; Marr et al. 1999; McGee et al. 2002), pH (Pagenkopf 1983; Welsh et al. 1993; Cusimano et al. 1986; Erickson et al. 1996; Taylor et al. 2002), alkalinity (Chakoumakos et al. 1979), and Na (Erickson et al. 1996) are probably the most important modifiers of toxicity during acute waterborne Cu exposure to fish. Increases in all of the above factors generally tend to decrease Cu toxicity; hardness (Ca²⁺ and Mg²⁺) and Na⁺ offer protection by competition with Cu²⁺, organic carbon and alkalinity by complexing Cu²⁺, and pH and alkalinity by changing Cu speciation away from Cu²⁺ to the less bioavailable species, CuCO₃ (aq) and CuOH⁻. Life stage (Howarth and Sprague 1978) and fish species or strain (Brix...
Figure 4. Schematic representation of Cu homeostasis in rainbow trout. Copper homeostatic mechanisms in trout maintain plasma (extracellular) Cu levels of 0.6-0.8 µg/mL, which satisfies the cellular requirements. Putative Cu sensors possibly located within local tissues, the central nervous system or the circulatory system could be responsible for monitoring Cu levels in plasma/extracellular fluid. The effector organs are primarily the liver, gut, and gill but other organs including the kidney may play some less important roles. Modulation of uptake and excretion maintains physiological levels of Cu in plasma and with body cells. + Indicates enhancement of physio-toxicological response.

et al. 2001, Meyer et al. 2002; Taylor et al. 2003) all significantly influence Cu toxicity. The decline in Cu uptake and gill binding with fish size (Kamunde et al. 2001; Taylor et al. 2002) helps explain the lower sensitivity to Cu observed with increase in fish size (Howarth and Sprague 1984). Because of the above-mentioned variables, 48- and 96-h LC50s for Cu vary by more than an order of magnitude (Meyer 1999).

Aquatic toxicologists are in agreement that acute toxicity of waterborne Cu results from impairment of Na and Cl homeostatic mechanisms (Wood 2001). Freshwater fish maintain blood NaCl levels >150 times the external environment and compensatory active NaCl uptake to replace NaCl lost down the concentration gradient is a key prerequisite for survival of fish in freshwater. At exposure levels of 12 to 2000 µg/L (depending on water chemistry, species, and life-stage), Cu reduces plasma and whole body Na and Cl (Stagg and Shuttleworth 1982; Laurén and McDonald 1985, 1986, 1987a; Wilson and Taylor 1993; McDonald and Wood 1993; Simkiss and Taylor 1995; Wood 2001). As Cu concentration increases, the first toxic effects are seen on active Na uptake. Copper impairs active Na uptake by competing with Na for the apical entry pathway (ENaC) and later by inhibiting branchial Na,K-ATPase at the basolateral membrane (Lorz and McPherson 1976; Laurén and McDonald 1987b; Beckman and Zaugg 1988; Pelgrom et al. 1995; Li et al. 1996; 1998; Grosell and Wood 2002). Impairment of Cl uptake possibly occurs indirectly via inhibition of carbonic anhydrase (Grosell et al. 2002), which provides the substrate for the Cl/HCO₃⁻ exchanger and couples Na and Cl uptake. Nonetheless, studies using rainbow trout erythrocytes have demonstrated direct competition of Cu and Cl for the Cl/HCO₃⁻ exchanger (Bogdanova et al. 2002). At higher levels, generally above 100 µg/L, Cu exposure increases diffusive Na⁺ efflux, presumably following Cu-induced displacement of Ca from the intercellular tight junctions (Laurén and McDonald 1986; Wood 2001) since Ca is thought to regulate paracellular permeability (Loretz 1987). In addition, studies using human intestinal Caco-2 cells showed that Cu increases tight junction permeability through an intracellular mechanisms involving perturbation of F actin cytoskeleton (Ferruzza et al. 1999). More recently, Grosell et al. (2002) suggested that impairment of carbonic anhydrase would secondarily interfere with N-excretion because of the linkage of N-excretion to Na transport (ie. Na⁺/NH₄⁺ exchange; Figure 3). Additional effects of acute toxicity of waterborne Cu in freshwater fish include reduction in arterial O₂ tension (PₐO₂), haemoconcentration, increase in arterial pressure, tachycardia, increased plasma ammonia and glucose, increased extracellular fluid pH, reduced swimming performance and increased cortisol production (Waiwood and Beamish 1978; Laurén and McDonald 1985; Wilson and Taylor 1993; Beaumont et al. 1995; Pelgrom et al. 1995). These effects occur at relatively high concentrations of waterborne Cu and are not Cu-specific. Several morphological changes including epithelial cell swelling, haematomas, cell death, and thickening and curling.
of gill lamellae (Kirk and Lewis 1993; Wilson and Taylor 1993; Bury et al. 1998; Li et al. 1998) are also evident during acute waterborne Cu exposure. Net Na⁺ and Cl⁻ loss across the gills triggers the chain of events leading to death from acute Cu exposure (Figure 5), in a similar manner to the effects of low pH exposure (Wood 1989) and Ag exposure (Hogstrand and Wood 1998). The toxic threshold is about a 30% loss of whole-body sodium for rainbow trout (Laurén and McDonald 1985; Grosell et al. 2002; Taylor et al. 2003) and 40% for yellow perch (Taylor et al. 2003). According to Grosell et al. (2002) smaller animals are more sensitive to Cu because they have higher Na turnover rates than large animals.

Chronic toxicity of waterborne copper in freshwater fish
An aspect of Cu that continues to elude toxicologists is the mechanism of chronic toxicity. Understanding the chronic effects of waterborne Cu exposure is complicated by the fact that fish may become acclimated (Dixon and Sprague 1981) and better suited to live at elevated Cu levels (McDonald and Wood 1993; Wood 1999). One possible way that chronic waterborne Cu toxicity can occur is via increase in plasma and extracellular Cu concentrations following breakdown of homeostatic mechanisms, leading to oxidative cell damage as witnessed in Wilson’s disease, a genetic disease of Cu metabolism in humans that manifests as toxicity (Schaefer and Gatlin 1999). We have indeed recently demonstrated that waterborne Cu toxicity is associated with elevated plasma Cu concentration (Kamunde et al. unpublished). Mechanisms not withstanding, chronic toxicity of Cu in fish may manifest as reduced growth and aerobic scope, loss of appetite, ion loss and even as mortality (Wairwood and Beamish 1978; De Boeck et al. 1995; Taylor et al. 1996; McGeer et al. 2000a). Full characterization of chronic toxicity will require the identification of internal effects concentrations at specific metal-sensitive sites/tissues, and the most sensitive chronic endpoint.

Toxicity of dietary copper
Until recently, dietary exposures of Cu to fish were largely ignored because gastrointestinal uptake was not considered an important pathway for Cu toxicity. Most dietary studies were therefore concerned with establishing a nutritional requirement for Cu in fish (Ogino and Yang 1980; Murai et al. 1981; Lorentzen et al. 1998), while toxicological studies focused primarily on the waterborne exposure pathway. It has now been recognised that the toxicology of Cu in aquatic environments is complex, and food may contribute substantially to total Cu uptake, and potentially to chronic toxicity (Miller et al. 1993; Handy 1996; Clearwater et al. 2002; Campbell et al. 2003). Specifically, in contaminated waters of high complexing capacity, dietary uptake and toxicity of Cu is probably predominant because of diminished uptake from the dissolved phase. However, dietary Cu levels even four orders of magnitude higher than those acutely toxic during waterborne exposure are apparently non-toxic. For example infusing a 270-g trout via a stomach catheter with a Cu dose as high as 30 000 µg (Clearwater et al. 2000) did not cause mortality in 4 h, neither did long-term feeding of a 10 000 µg/g Cu diet (Handy 1993), in part due to the fact that fish either reject (food) or regurgitate the Cu soon after ingestion/infusion. It is therefore unlikely that dietary Cu is acutely toxic to fish, especially because environmentally realistic levels are relatively low (10 – 1800 µg/g dry weight in invertebrates [Dallinger and Kautzky 1985; Handy 1996]).

Chronic toxicity of dietary Cu has been variably demonstrated in freshwater fish (Clearwater et al. 2002) but to date the toxic concentration of Cu in the food remains controversial. Generally, indications are that toxicity of dietary Cu will depend on the species and life stage of the fish and the form in which it is presented to the fish (Clearwater et al. 2002). While using dietary Cu concentrations to determine the toxic levels of Cu yields very variable levels of toxic threshold (Kamunde et al. 2001), a recent synthesis of data on dietary Cu exposure in fish shows that a daily dose 35-45 µg Cu per g fish is toxic to rainbow trout (Clearwater et al. 2002). Thus Clearwater et al. (2002) argue that the dose (which takes into account the feeding rate and the concentration of the metal in the food),
rather than the metal concentration in the food/prey alone should be used to determine toxic levels of Cu in food. In agreement with this sentiment, Kamunde and Wood (2003) independently came to the conclusion that using total dose as a measure of exposure is more appropriate because fish fed a constant dose of Cu in different rations accumulated very similar amounts of Cu in their bodies (i.e. Cu/fish) despite very different concentrations on a per unit mass basis.

The mechanisms of dietary Cu toxicity likely reflect a suite of chronic effects. Some of these may stem from generation of free radicals in the tissues where Cu accumulates and manifest as lipid peroxidation, e.g. in the liver, gut, and kidney (Farag et al. 1994, 1995; Baker et al. 1998), while others may result from inhibition of gut motility and digestive enzymes (Woodward et al. 1995), degeneration of gut epithelium (Handy 1993; Farag et al. 1999), decreased whole fish oxygen consumption (Handy et al. 1999) and increased gastrointestinal cell turnover via apoptosis (Berntssen et al. 1999; Kamunde et al. 2001). Although some studies reported growth impairment following dietary Cu exposure (Murai et al. 1981; Lanno et al. 1985; Woodward et al. 1994, 1995), several recent studies have shown no effects (Gatlin and Wilson 1986; Mount et al. 1994; Berntssen et al. 1999; Handy et al. 1999; Kamunde et al. 2001, 2002a,b; Kamunde and Wood 2003) suggesting low bioenergetic cost of regulating dietary Cu in fish. Interestingly, gastrointestinal Na⁺,K⁺-ATPase was unaffected after 35 days of exposure of rainbow trout to 15 µg/g fish/day (Kamunde and Wood 2003), suggesting that either the dose was non-toxic or that the mechanisms of dietary Cu toxicity differ from those of acute waterborne Cu exposure.

**Acclimation**

During prolonged exposure to elevated waterborne Cu, fish exhibit physiological recovery and increased tolerance to subsequent acute Cu challenge, a phenomenon termed acclimation, which was first demonstrated in a rigorous toxicological study by Dixon and Sprague (1981). Figure 6 illustrates the potential effects of acclimation on toxicity and optimal levels of Cu in fish. Generally, acclimation can be viewed as the net result of two processes, one leading to the recovery of physiological disturbance, and the other to the regulation of uptake and distribution of Cu. In the first process, fish under sublethal Cu stress exhibit an initial disturbance of Na homeostasis followed by recovery, in the so-called damage-repair hypothesis (McDonald and Wood 1993).McGeer et al. (2000a) demonstrated a classical damage-repair hypothesis (McDonald and Wood 1993). McGeer et al. (2000a) demonstrated a classical damage-repair hypothesis. During a chronic exposure of rainbow trout to 75 µg/L waterborne Cu, in which disturbance in Na homeostasis in the first two days had fully recovered within a week. These authors attributed the return to normal of the physiological responses to the recovery of the Na⁺,K⁺-ATPase activity, following synthesis of more enzyme units; enzyme activity was more than two times the control activity after 60 days of exposure. Apparently the diffusive loss of Na was effectively counteracted by greater Na uptake due to higher Na⁺,K⁺-ATPase activity. A second mechanism by which Na homeostasis recovers...
Environmental regulation of copper

Kamunde and Wood

is likely via the reduction of paracellular pathway permeability since a specific toxic action of Cu is to increase the permeability of intercellular junctions, leading to increased diffusive Na loss (Laurén and McDonald 1987b). As well, since Cu exposure causes morphological damage to the gill epithelium (Kirk and Lewis 1993; Wilson and Taylor 1993; Bury et al. 1998; Li et al. 1998), a third mechanism of recovery is likely associated with the repair of epithelial structural damage. Finally, conservation of NaCl by the kidney (Grosell et al. 1998b) offers an additional method through which Na homeostasis can be reclaimed.

The second facet of acclimation involves regulating Cu accumulation in the animal, which can occur at the uptake, distribution, and storage phases of Cu toxicokinetics. Although decreased uptake into the whole body and/or gill has been reported (Grosell et al. 1997; Kamunde et al. 2002a, Gale et al. 2003), no change (McCarter and Roch 1984; Grosell et al. 1996), and increased uptake (Taylor et al. 2000; McGee et al. 2002) have also been reported. Kamunde et al. (2002a) explained these variant responses to acclimation to waterborne Cu on the basis of differing effects of Cu on the “high-affinity low-capacity” (HALC) and the “low-affinity high-capacity” (LAHC) Cu binding sites on the gills (Taylor et al. 2000). While Cu uptake via the HALC binding sites is reduced by acclimation, uptake via LAHC binding sites is increased such that the measured response will depend critically on the ambient Cu concentration during the Cu flux measurement (Kamunde et al. 2002a). The second strategy by which fish regulate Cu is by increasing excretion via the hepatobiliary, branchial, and gastrointestinal pathways (as detailed in Excretion of copper). The third option for tolerance induction is increased storage, detoxification or sequestration of Cu in specific organs or cell compartments where it is unavailable to sensitive cellular components. To this end, Cu binding proteins and bio-molecules, eg. metallothionein, acid-soluble thios and glutathione, are induced in fish tissue following chronic sublethal exposure to Cu (McCarter and Roch 1984; Laurén and McDonald 1987b; Marr et al. 1995; Dang et al. 1999, 2000; Handly et al. 1999). Copper held in these proteins/bio-molecules is presumably less labile (Kamunde et al. 2002a) and therefore less toxic. Finally, repair of damaged gill epithelium, as noted above, also mitigates the continued uncontrolled influx of Cu, in addition to limiting paracellular influx of Na.

Increased tolerance to waterborne Cu following exposure to dietary Cu has also been reported (Miller et al. 1993), but Kamunde and Wood (2003) noted only insignificant trend toward increased waterborne LC50 in rainbow trout exposed to 15 µg Cu/g fish per day, possibly because the dose was much lower relative to the Miller et al. study. Surprisingly, while acclimation to waterborne Cu downregulates waterborne Cu uptake, dietary Cu uptake is unaffected (Kamunde et al. 2002a). Studies to assess the effect of acclimation to dietary Cu on subsequent uptake of dietary Cu need to be carried out.

Environmental regulation of copper

Many governments throughout the world recognise the potential hazard posed by elevated levels of metals such as Cu in the environment. However, introduction of measures to regulate the release of metals into the environment is constrained by a lack of adequate knowledge on the effects of metals on aquatic organisms, particularly in relation to the normal chemical and nutritional environment of the organisms. As a result of these scientific limitations, policy analysts often employ the concept of Precautionary Principle as a means of taking anticipatory action in the absence of satisfactory scientific proof of harmful effects. However, inappropriate application of the Precautionary Principle may result in unnecessary bans on the use of metals, barriers to trade, blanket rather than site-specific regulations, inappropriate targeting of resources for environmental protection, and environmental regulations levels written below nutritional or analytical detection limits. These potential problems have resulted in scepticism in acceptance of regulatory programs by industry because of the potential for overprotection coupled with the rigidity of the regulatory system (Paquin et al. 2002). In aquatic toxicology the most effective antidote to inappropriate application of the Precautionary Principle is sound scientific knowledge.

In the U.S., water quality criteria are now based on dissolved Cu (not total Cu) and a correction for hardness is incorporated (USEPA 1993), based on improvements in knowledge made over the last three decades. However, it is now clear that dissolved metal is not a good predictor of bioavailability and toxicity (Bergman and Dorward-King 1997; Jensen et al. 2000), because it does not account for metal bound to Dissolved Organic Carbon (DOC), which is unavailable, nor does it consider other factors that affect Cu bioavailability and toxicity such as pH, alkalinity, and Na levels. Consequently, adjustments using the concept of the water-effect ratio (WER) were introduced (USEPA 1994), and thus bioavailability-related modifications of WQC without adequate understanding of site-specific modifying factors were made possible (Bergman and Dorward-King 1997). In brief, the WER ratio experimentally compares the toxicity of Cu in the site-specific water with that in the “laboratory water”, and may result in a less stringent regulation when the site-specific water offers significant protection. However, the WER was found to be inefficient for application to WQC adjustments on a wide scale (USEPA 2001) because it entails large-scale animal testing (therefore time-consuming and expensive), necessitating the development of improved methodologies to better characterise natural exposure scenarios. The Biotic Ligand Model (BLM) approach, which is a computational method based on sound science, offers great promise in efficiently deriving realistic and widely acceptable criteria while limiting the use of the Precautionary Principle.

The Biotic Ligand Model: an antidote to application of the Precautionary Principle

The BLM was developed by a consortium of U.S. and Canadian researchers as a predictive tool for acute toxicity of waterborne metals to fish (Di Toro et al. 2001; Santore et al. 2001; Paquin 2002) and is gaining wide acceptance among the scientific, regulatory, and regulated communities. The BLM is based on understanding of physiology, toxicology, and geochemistry, and uses known receiving water chemistry, “Biotic Ligand”(gill) metal binding constants, and standard geochemical modelling programs
(eg. CHESS, MINEQL+, MINTEQA2, WHAM) to predict acute toxicity. Although the BLM concepts were first developed under laboratory conditions with a limited number of species (eg. Playle et al. 1993a,b; Playle 1998; MacRae et al. 1999), the versatility of the BLM has resulted in its provisional approval (USEPA 2001) for site-specific modification to Cu Acute Criteria. The greatest advantages of the BLM are that it is site-specific, cost-effective, avoids animal testing, and has potential application for both acute and chronic criteria for Cu and other metals including Ag, Cd, Zn, Pb, and Ni. It has been argued that implementation of the BLM approach will likely reduce the need for application of the Precautionary Principle, identify situations in which regulation has been inappropriate, and reduce the costs for implementation of regulation (Paquin et al. 2002).

Recent advances in the development and application of the BLM for WQC derivation and ERA of metals have been summarised (Gorsuch et al. 2002; Niyogi and Wood 2003). Because the BLM is a computational model, new developments can be easily incorporated, and the model can continue to evolve as new findings are made. Here we highlight recent developments of the Cu BLM, with special emphasis on features of the biotic ligand (gill) and factors influencing its capacity to bind Cu. A conceptual model of the Cu BLM is shown in Figure 7. This model captures the complexation reactions with ligands in the water that bind Cu as well as competition reactions with waterborne cations for active binding sites at the gill surface. Toxicity occurs when short-term metal accumulation (generally at 3-24 h of exposure) at the gill active sites reaches a lethal threshold, for example, an accumulation that causes 50% mortality by 96 h is described as the 96-h LA50 (lethal accumulation). For Cu, the main ligands influencing bioavailability and toxicity are DOC, CO$_3^{2-}$, and OH$^-$ and the main cations are Ca$^{2+}$, H$^+$, Mg$^{2+}$, and Na$^+$. The binding capacity, or binding site density (B$_{max}$) and the affinity constant (log K) are both generally determined using short term (3 h) in vivo gill metal binding assay to minimise metal export from gill and possible physiological changes to the binding sites. Earlier researchers characterised a single gill Cu binding site (Playle et al. 1993a, MacRae et al. 1999) with log K and B$_{max}$ values ranging from 7.1 to 7.6 and from 30 to 63 nmol/g, respectively. However, recent studies using a more sensitive radioisotope methodology identified two Cu binding sites, designated HALC and LAHC Cu binding sites (Taylor et al. 2002, 2003, 2004; Kamunde et al. 2002a). The high affinity (HALC) sites have Cu-gill log K values of 7.9 to 9.2 and are likely the real site of interest for the acute BLM since they saturate at environmentally realistic Cu levels, while the high capacity (LAHC) sites are unsaturable and less relevant for BLM purposes. The mixture of the two types of sites probably results in the lower operative log K values (7.1 to 7.6). The actual active sites of toxic action of waterborne Cu (Figure 3) probably include ENaC on the apical membrane of the gill (Grosell and Wood 2002; Kamunde et al. 2003, Pyle et al. 2003) and the basolateral Na$^+$,K$^+$-ATPase (Laurén and McDonald 1987b; Pelgrom et al. 1995; Li et al. 1998); both proteins comprise the Na uptake mechanism in freshwater fish.

**Acclimation and BLM**

The modification of acute toxicity (ie. increase in LC50) by previous chronic exposure to Cu (acclimation) has been clearly demonstrated in several studies (Table 1; see also Acclimation). From a BLM perspective, acclimation reduces the affinity of HALC Cu binding sites and increases the capacity of the LAHC Cu binding sites (Taylor et al. 2000; Kamunde et al. 2002a). Moreover, acclimation potentially increases LA50 (Figure 6). The current Cu BLM

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**Figure 7. Conceptual model of the Cu BLM.**
The model describes toxicity in terms of interaction of Cu, the surrounding water and the gill surface. The toxic action is primarily driven by the concentration of Cu in the water. Competition for binding sites with cations present in the water and complexation of Cu by environmental ligands such as DOC modify toxicity (adapted from Paquin et al. 2002).
(Santore et al. 2001) does not consider the effects of acclimation on gill-Cu binding characteristics or toxicity; future refinement of BLM should therefore accommodate these effects. Indeed, a recent study using laboratory-reared fish and fish chronically exposed to Cu (and other metals) in metal-contaminated lakes in the Sudbury region, Ontario, Canada (Taylor et al. 2004) suggests that considering these effects increases the predictive precision of the acute BLM.

**Dietary variables and the BLM**

Another rapidly emerging area of BLM significance is the role of dietary variables such as food quality and quantity. Dietary variables are known to affect fish physiology but their role in the toxicity of metals has been largely ignored (Lanno et al. 1989). In fact, several effects of food-related variables on Cu uptake and gill binding characteristics have been documented in the last few years (Table 1; Kamunde et al. 2001, 2002b, 2003; Kamunde and Wood 2003; Niyogi and Wood 2003). Kamunde and Wood (2003) reported modest stimulation of gill Cu binding in rainbow trout maintained on high rations (3.0 or 4.5% wet body weight) relative to those maintained on a low ration (1.5% wet body weight). In several other studies (Kamunde et al. 2001, 2002b, 2003; Pyle et al. 2003) effects of dietary Cu and Na contents were assessed. The results obtained with chronic exposure to dietary Cu may have implications for the BLM in that pre-exposure to elevated concentrations of dietary Cu reduced subsequent uptake and binding of Cu to gills (Kamunde et al. 2001; 2002b) and induced tolerance (increased LC50) to waterborne Cu (Miller et al. 1993). On the other hand, exposure to dietary Na reduced binding of Cu to gill (Kamunde et al. 2003, Pyle et al. 2003) and suggested a reduction in the affinity of Cu binding to the gill. The dietary Na levels used were only a few times greater than those found in the natural diet of fish. Interestingly, elevated dietary Ca has a parallel effect, reducing binding of cadmium (a Ca analogue), on trout gills (Zohouri et al. 2001; Baldisserotto et al. 2003). It is possible that fish in Cu and/or Cd impacted environments may select prey richer in Na and/or Ca to mitigate the effects of metals exposure. Indeed it has been shown that fish have the ability to select prey in nature. For example when offered choice, fish select larger prey (Lazzaro 1987).

Differentiation of effects of dietary metal pre-exposure from acclimation to elevated waterborne Cu concentrations will not be an easy task. However, the BLM coupled to kinetic modelling might be useful for this purpose. We envisage a two-tiered approach whereby kinetic modelling would initially delineate the principal pathway of exposure at site-specific conditions. When water is shown to be the main pathway of uptake and other factors have been accounted for, discrepancies between BLM predicted values and determined values would likely mean that BLM characteristics have been modified by chronic exposure to waterborne Cu. Conversely, when diet is the main pathway, discrepancies between determined and predicted values would be interpreted as modification of binding characteristics by a dietary variable such as exposure to elevated Cu or Na levels in the food.

**A chronic BLM**

Recently, regulatory authorities throughout the world (eg. in North America, Europe, and Australia/New Zealand) have become much more interested in the development of chronic WQC designed to protect against deleterious effects of long-term sublethal exposure so as to provide life-long protection to natural populations. Potentially, a chronic BLM for Cu could be developed on the same principle as the current acute BLM by relating chronic endpoints (health, performance, growth, reproduction) to tissue specific Cu burdens. The need to develop a chronic BLM for Cu is vital in view of the fact that at present, chronic WQC are invariably derived using the acute-to-chronic ratio (ACR), an extrapolation based on the flawed assumption that the mechanisms of acute and chronic toxicity are the same. Recently, Niyogi and Wood (2003) presented a comprehensive review of BLM application in ERA, in which the need to consider temporal and spatial dynamism of the gill–metal interactions (ie. changes as a result of other variables or prior exposure) in the future development or refinement of BLM was articulated. Given that such changes occur during chronic exposures to metals, research to better understand metal homeostasis, as well as to identify the most relevant chronic endpoints of toxicity (eg. growth often proves to be very insensitive), is necessary. Other key issues that need to be untangled in future development of the chronic BLM include how to deal with assumptions of the model, such as biological effects being constant at a specified accumulation of metal in the organism, and cell characteristics remaining un-altered during metal-cell binding because these assumptions are not always met. In addition, the most appropriate methods by which to make adjustments for species sensitivity (Brix et al. 2001; Meyer et al. 2002; Taylor et al. 2003) need to be considered in further development of the BLM.

**CONCLUSION**

The apparent shift in regulatory policy by USEPA toward greater emphasis on chronic toxicity than acute toxicity, which for many years has been the regulatory focus in Canada (CCME 1999), Europe (EC 1993), and Australia/New Zealand (ANZECC and ARMCANZ 2000), suggests that many of the world’s leading environmental regulatory bodies now have a common policy, albeit different in detail. However, to model chronic toxicity for site-specific WQC derivation, for example by a BLM type approach, requires a good grasp of Cu homeostasis since chronic toxicity probably results from the breakdown of homeostasis. Clearly, bioavailability, waterborne and dietary exposures and their relative importance under site-specific conditions, acclimation, and physiological homeostasis, all need to be considered in setting realistic WQC. It is particularly important to determine if food is a significant source of Cu before relying on uptake from water only to model toxicity. Accumulation of Cu in tissues other than the gill will likely assume greater importance since the role of the gill in chronic toxicity may be somewhat diminished. However, the identification of sensitive endpoints for chronic toxicity and the most relevant tissues to consider remains a major scientific challenge in aquatic toxicology. Based on the information contained in this review, it is clear that current approaches for WQC derivation and ERA, while far superior to those available several decades ago, still leave scope for considerable improvement. Incorporation of knowledge summarised here, as well as other recent relevant scientific data on bioavailability, toxicology, and homeostasis, will greatly improve the accuracy of the BLM approach and limit the application of the Precautionary Principle in ERA and WQC derivation for Cu.
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Kamunde and Wood

18
Environmental regulation of copper


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