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Review

Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals☆

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Abstract

The mechanisms of acute copper and silver toxicity in freshwater organisms appear similar. Both result in inhibition of branchial sodium (and chloride) uptake initiating a cascade of effects leading to mortality. The inhibition of the branchial Na/K-ATPase in the basolateral membrane is generally accepted as the key component responsible for the reduced sodium uptake. We propose that branchial carbonic anhydrase and the apical sodium channel may also be important targets for both copper and silver exposure. Several attempts have been made to predict metal sensitivity. A prominent example is the geochemical–biotic ligand model. The geochemical–biotic ligand modeling approach has been successful in explaining variations in tolerance to metal exposure for specific groups of animals exposed at different water chemistries. This approach, however, cannot explain the large observed variation in tolerance to these metals amongst different groups of freshwater animals (i.e. Daphnia vs. fish). Based on the detailed knowledge of physiological responses to acute metal exposure, the present review offers an explanation for the observed variation in tolerance. Smaller animals are more sensitive than large animals because they exhibit higher sodium turnover rates. The same relative inhibition of sodium uptake results in faster depletion of internal sodium in animals with higher sodium turnover. We present a way to improve predictions of acute metal sensitivity, noting that sodium turnover rate is the key predictor for variation in acute copper and silver toxicity amongst groups of freshwater animals. We suggest that the presented sodium turnover model is used in conjunction with the Biotic Ligand Model for risk management decisions.

Keywords: Copper; Silver; Freshwater; Fish; Crustaceans; Sodium transport; Ammonia excretion; Predicting mortality

1. Introduction

When present in elevated concentrations in the water, both silver and copper cause toxicity to aquatic organisms with the ionic forms of these metals generally being most toxic. Consequently, the emission of these metals into the environment is currently under regulation. Current (since 1995) regulations are largely based on dissolved metal in effluents and/or in the environment but some parameters that protect against the effects of metals have been taken into account. For several metals, including copper, the US-EPA's current water quality criteria corrects for water hardness (USEPA, 1985), which offers some protection against acute silver and copper exposure (Pagenkopf, 1983;

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Erickson et al., 1987, 1996, 1998). In addition to hardness, other water chemistry parameters ameliorate the effects of acute exposure to these metals. The effects of acute exposure to copper are influenced by pH (Pagenkopf, 1983; Cusimano et al., 1986; Erickson et al., 1996; Welsh et al., 1993) and dissolved organic matter (Brown et al., 1974; Erickson et al., 1996; Welsh et al., 1993). As for copper, the acute effects of silver exposure can also be reduced by dissolved organic carbon (Bury et al., 1999a,b; Erickson et al., 1998). Chloride also offers a clear protective effect against acute silver toxicity to rainbow trout (*Oncorhynchus mykiss*) (Galvez and Wood, 1997; Bury et al., 1999a,b) but not to fathead minnow (*Pimephales promelas*) (Erickson et al., 1998; Karen et al., 1999; Bury et al., 1999b) or the European eel (*Anguilla anguilla*) (Grosell et al., 2000). Thiosulphate and reactive sulphide clusters clearly reduces acute silver toxicity to freshwater organisms (Wood et al., 1996b; Bianchini et al., 2002a).

The protective effects of various water chemistry parameters have been modeled in the Biotic Ligand Model (BLM) for silver (Paquin et al., 1999) and copper (Santore et al., 1999, 2001). The BLM simultaneously accounts for the speciation and complexation of dissolved metal and competitive binding of metal and other cations at the site of action, the gill. The premise behind the BLM is that there is a strong correlation between the metal concentration in/on the target and the subsequent acute toxicity. While such a correlation has been documented for copper (MacRae et al., 1999; Santore et al., 1999, 2001), it seems less certain for silver (reviewed by Wood et al., 1999). The variable levels of both silver (Bury and Wood, 1999; Wood, 2001) and copper (Grosell et al., 1997; Grosell and Wood, 2002) in the gills of rainbow trout during continued exposure suggests that some caution should be taken when attempting to model toxic responses based on predicted gill metal concentration as it is currently done in the BLM. Recently, McGeer et al. (2000) presented a physiologically based BLM that successfully predicted acute silver toxicity in rainbow trout using branchial Na/K-ATPase inhibition as an endpoint for silver toxicity. This report illustrates the significance of a detailed understanding of the mechanism of toxicity when attempting to predict effects.

The BLM is largely based on data obtained from fish and is currently calibrated to protect invertebrates that are much more acutely sensitive to both silver and copper based on toxicity data for *Daphnia* spp. (Paquin et al., 1999; Santore et al., 1999, 2001). The extrapolation from fish to invertebrates must rely on the general assumption that the mechanisms of copper and silver induced toxicity in the highly sensitive invertebrates are the same as those in the less sensitive teleost fish. However, this assumption remains to be tested and the reason for the large differences in sensitivity (often several orders of magnitude) to copper and silver between freshwater invertebrates and fish is unknown.

The purpose of the present review is to summarize the current knowledge of the toxic mechanisms of silver and copper to freshwater organisms to date and to offer a possible explanation for the large variation in sensitivity observed among freshwater animals. As a background, gill physiology relevant for acute copper and silver toxicity is briefly reviewed. The literature review for the present paper was completed by October 1st 2001.

2. Branchial sodium and chloride transport in freshwater animals

The gill is a multi-functional organ with a very complex anatomical structure. It is generally accepted that the freshwater gill serves many purposes such as respiration, nitrogenous waste excretion, acid–base balance and osmoregulation. More recently, it has been documented that the gill, at least in rainbow trout, serves a role in trace element absorption (Spry et al., 1988; Kamunde et al., 2002).

The general response to toxicants includes nonspecific structural changes, which in turn may influence general gill functions including respiration. However, the present review, will focus on the more specific physiological responses reported from freshwater animals during acute exposure to lower concentrations of silver and copper. For additional information, refer to Wood (2001) for a comprehensive review of the toxic response of gill structure and function in fish.

The key target for both copper and silver in freshwater fish appears to be sodium homeostasis, although chloride absorption and nitrogenous waste excretion can also be influenced. Sodium uptake from the water across the gills is essential for any water breathing freshwater animal as it serves to compensate for the diffusive loss of sodium from its concentrated extracellular fluids (extracellular sodium concentrations in the 100–

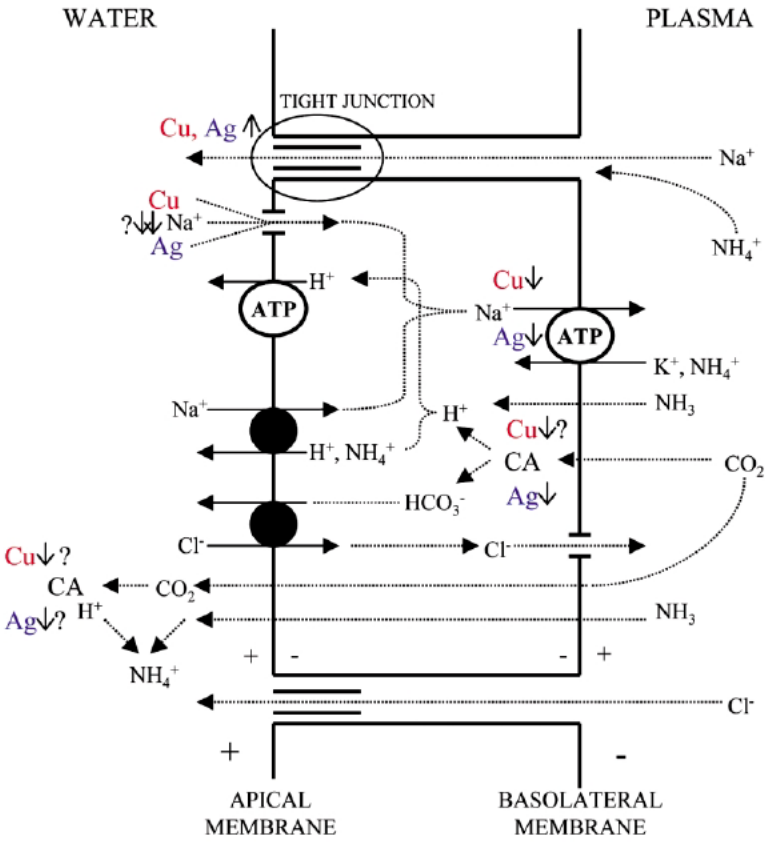

Fig. 1. Schematic representation of a general model of acid–base, sodium, chloride, NH_3^+ and NH_4^+ transport across the branchial epithelium of freshwater organisms. The depicted transport processes may well occur in different cell types, but since this is unresolved at present, the diagram should be considered as overall branchial transport regardless of cell type. The '+' and '−' indicate the direction of membrane and epithelial potentials. Dotted lines represent diffusive transport events, while solid lines depict all other events. The carriers marked with 'ATP' perform active transport dependent on the dephosphorylation of adenosine triphosphate (ATP). Carbonic anhydrase is abbreviated CA. The symbols '↑' and '↓' denote that the metal (Ag and/or Cu) increases or decreases the relevant process, respectively.

200 mM range) to the surrounding dilute environment (typical sodium concentration <1 mM). Fig. 1 summarizes the current understanding of sodium transport across the freshwater gill epithelium. For the sake of simplicity, only components of sodium and directly associated transport processes are depicted. Possible ammonia transport pathways are included in the model since they may in some way be linked to sodium transport and they appear to be sensitive to both copper and silver exposure. Sodium and chloride transport may occur through different cell types (pavement cells versus chloride cells), however, this remains controversial. The transport events illustrated in Fig. 1 should be considered a diagram of the overall branchial transport regardless of cell type.

One principle component of epithelial transport processes is the Na/K-ATPase located in the basolateral membrane. This membrane bound enzyme was first identified by Skou (1957) and exchanges intracellular sodium for extracellular potassium in a 3:2 ratio. This ATP-dependent electrogenic exchange polarizes the intra-cellular compartment (see Skou, 1990, Skou and Esmann, 1992, for reviews) thereby setting up an electrochemical gradient favoring sodium influx across the apical membrane. Sodium entry across the apical membrane appears to be in exchange for protons or ammonia (Krogh, 1938) via either a sodium–proton exchanger or a sodium channel coupled to a proton pump (Potts, 1994). Both these sodium–proton exchange mechanisms have

been included in Fig. 1 but the proton pump/sodium channel hypothesis seems to be more accepted at least in freshwater fish (Fenwick et al., 1999; Bury and Wood, 1999; Perry et al., 2000; Grosell and Wood, 2002). The extrusion of protons across the apical membrane via the proton pump generates an electrochemical gradient that favors sodium entry through the apical sodium channel. Molecular evidence for the presence of the proton pump has been given by Sullivan et al. (1995, 1996) and Perry et al. (2000), while pharmacological evidence for the involvement of both the proton pump and the sodium channel in branchial sodium uptake in rainbow trout has been reported by Fenwick et al. (1999), Bury and Wood (1999) and Grosell and Wood (2002) and in crayfish by Zetino et al. (2000). It should be noted that several isoforms of sodium–proton exchangers have been identified in gills of seawater and euryhaline fish (Claiborne et al., 1999; Wilson et al., 2000) and thus could be present in gills of freshwater animals. Indeed, Wood and Pärt (1996) demonstrated that intracellular pH in rainbow trout pavement cells is regulated by a sodium–proton antiport mechanism.

Regardless of the mechanism of apical sodium uptake, Na^+ entry occurs in exchange for protons. These protons arise from the hydration of carbon dioxide by cellular carbonic anhydrase which facilitates the conversion of carbon dioxide to protons and bicarbonate (Perry, 1986) and thus fuels the proton extrusion and the sodium uptake. In addition, carbonic anhydrase provides cellular substrate for the chloride–bicarbonate exchange that occurs across the apical membrane, excreting bicarbonate in exchange for chloride uptake. The functional importance of this carbonic anhydrase mediated chloride uptake by trout gills was first demonstrated by Perry et al. (1984). While it is generally accepted that chloride uptake across the apical membrane occurs via a chloride–bicarbonate exchanger (Wood, 2001), the mechanism of chloride extrusion across the basolateral membrane remains unknown. A basolateral CFTR-like chloride channel (cystic fibrosis transmembrane regulator) has been identified in fish gills (Marshall et al., 1995, 1999; Singer et al., 1998) and could serve this purpose. The uptake of sodium and chloride is thus coupled indirectly via cellular carbonic anhydrase.

3. Ammonia excretion across freshwater fish gills

It is clear that the primary elimination of nitrogenous waste in most freshwater fish occurs as ammonia/ammonium excretion across the gills, however, the mechanisms involved in this process remain unsettled (see Wood, 1993; Wilkie, 1997, for reviews). There seems to be a general consensus that a diffusive component to both NH_3 (ammonia) and NH_4^+ (ammonium) excretion exists (Wood, 2001) but also the early suggestion of sodium–ammonium exchange across the apical membrane (Krogh, 1938) has some support. In addition to simple diffusion of ammonia, ammonium can enter the gill cells from the blood via the basolateral Na/K-ATPase where ammonium can displace potassium (Mallery, 1983; Towle and Hølleland, 1987; Wall and Koger, 1994). The involvement of the basolateral Na/K-ATPase in ammonium transport is supported by findings of reduced ammonia/ammonium (T_{amm}) excretion by ouabain in trout gills (Payan, 1978). The ammonium ion could be extruded across the apical membrane via a sodium exchange (Randall et al., 1999; Wilson et al., 2000) at least in some fish species as suggested first by Krogh (1938). An appealing hypothesis of carbonic anhydrase dependent diffusion trapping of ammonia has been presented (Randall and Wright, 1989; Wright et al., 1989; Wilson et al., 1994). This model relies on the instant conversion of ammonia to ammonium by the protons in the water boundary layer of the gill surface, thus maintaining very low concentrations of ammonia and thereby favorable conditions for ammonia diffusion across the apical membrane (termed 'diffusion trapping'). The water boundary layer could be acidified by hydration of excreted carbon dioxide via carbonic anhydrase found externally on the apical surface of the gill (Rahim et al., 1988; Wright et al., 1986) and/or by apical sodium–proton exchangers and/or proton pumps (see above). The latter proton extrusion across the apical membrane is linked to cellular carbonic anhydrase, as discussed above, and it thus appears that regardless of the sources of boundary layer acidification, it depends on the activity of either external or intracellular carbonic anhydrase. This points to a strong link between carbonic anhydrase and ammonia excretion across the gills of freshwater organisms.

4. Acute effects of copper and silver on gill physiology

4.1. Coppers effect on sodium and chloride homeostasis

Copper, although essential has been recognized as potentially toxic and induced disturbance of sodium balance in aquatic organisms was first demonstrated by Holm-Jensen (1948) in *Daphnia magna* exhibiting reduced whole body sodium after a few hours of exposure. Later findings of reduced plasma osmolarity, chloride and sodium concentrations in various fish species exposed to copper in freshwater confirmed that this metal was an osmoregulatory toxicant (McKim et al., 1970; Lewis and Lewis, 1971; Christensen et al., 1972; Schreck and Lortz, 1978; Stagg and Shuttleworth, 1982). It was believed that copper possibly targeted sodium and chloride transport systems in the gills of freshwater animals. In the pioneer work by Laurén and McDonald (1985), the reason for this copper-induced disturbance of sodium homeostasis in rainbow trout was determined to be a reduction in branchial sodium uptake at lower copper concentrations and a combination of reduced branchial sodium uptake and increased sodium loss at higher copper concentrations. The elevated sodium efflux across the gills of rainbow trout exposed to higher copper concentrations was attributed to displacement of calcium by copper in the tight junctions, which partly control the permeability of the branchial epithelium (Laurén and McDonald, 1985).

Inhibition of sodium influx, but not increased sodium efflux has since been reported from copper-exposed tilapia (*Orechromis mossambicus*) (Pelgrom et al., 1995). The inhibition of branchial sodium uptake in rainbow trout was subsequently determined to be the result of a mixed competitive (decreased affinity) and non-competitive (decreased maximal capacity) inhibition after 24 h of copper exposure (Laurén and McDonald, 1987a). These observations were paralleled by an inhibition of maximal branchial Na/K-ATPase activity leading to the conclusion that the inhibition of this enzyme during copper exposure was the reason for the observed reduction in branchial sodium uptake (Laurén and McDonald, 1987b). Parallel inhibition of sodium influx and branchial Na/K-ATPase in rainbow trout and tilapia during copper exposure has since been confirmed (Sola et al., 1995; Pelgrom et al., 1995) and the degree of inhibition of this enzyme and the copper concentration in the gill tissue has been reported to exhibit a positive correlation (Li et al., 1998; De Boeck et al., 2000). The inhibition of Na/K-ATPase appears to be through interference with magnesium binding to the enzyme (Li et al., 1996), which is critical for the phosphorylation that results in transport function (Skou, 1990).

A very recent study (Grosell and Wood, 2002) revealed that only 2 h of copper exposure at low concentrations causes a reduced branchial sodium transport affinity indicating competitive interaction between copper and sodium transport systems. This reduced transport affinity tended to increase the maximal sodium transport capacity rather than decrease it after 24 h of exposure as reported by Laurén and McDonald (1987a). Although no information exists on the detailed time course of copper induced Na/K-ATPase inhibition, it seems unlikely that a 2-h exposure is sufficient to effectively inhibit this enzyme. This assumption is based on reports of plasma copper levels in rainbow trout during copper exposure that did not reach maximum levels until 6 h of exposure (Grosell et al., 1997), suggesting that some time is required for copper to cross the apical membrane and reach the basolateral membrane (and affect the Na/K-ATPase) of the gill epithelium. This could indicate that other components of branchial sodium transport than the Na/K-ATPase could be influenced by acute copper exposure. In addition, the discrepancies between the effects of copper on branchial sodium transport kinetics from these two studies (constant or increased versus reduced maximal sodium transport capacity) may suggest that different systems are influenced depending on the duration of the exposure (2 vs. 24 h). Other components of branchial sodium uptake include apical sodium–proton exchangers and sodium channels and also intracellular carbonic anhydrase (see above). Inhibition of any of these components would influence sodium transport across the gills. Very strong bi-directional competitive interactions between sodium and copper uptake across trout gills (Grosell and Wood, 2002) indicate that the apical sodium channel could be a target for acute copper toxicity. In addition, inhibition of intracellular carbonic anhydrase would reduce branchial sodium transport since it would deplete a sodium–proton exchanger or the proton pump, both being sodium entry mechanisms using protons as sub-

strate. Although copper-induced inhibition of branchial carbonic anhydrase, to our knowledge, has yet to be demonstrated in freshwater animals, it is clear that carbonic anhydrase readily binds copper (Ditusa et al., 2001) and that copper can inhibit branchial carbonic anhydrase in the estuarine crab, *Chasmagnathus granulata* (Vitale et al., 1999).

No direct measurements of branchial chloride uptake during copper exposure in freshwater animals have been reported. However, several reports of reductions in plasma chloride levels similar to those observed in plasma sodium (Laurén and McDonald, 1985; Wilson and Taylor, 1993) strongly suggest that branchial chloride uptake, like sodium uptake, is impaired by copper. Indeed, copper potently inhibits chloride transport across the opercular epithelium, a model for the gill, of seawater adapted killifish, *Fundulus heteroclitus* (Crespo and Karnaky, 1983). Our current understanding of the transport physiology of the freshwater gill does not link chloride absorption to the Na/K-ATPase activity in the branchial epithelium but sodium and chloride transport is clearly linked by intracellular carbonic anhydrase (Fig. 1). Assuming that the parallel reduction in plasma sodium and plasma chloride seen during acute copper exposure (Laurén and McDonald, 1985; Wilson and Taylor, 1993) reflects a parallel inhibition of sodium and chloride uptake across the gills, intracellular carbonic anhydrase thus seems a likely target for acute copper toxicity.

5. Copper and ammonia/ammonium excretion

Copper exposure is often associated with elevated plasma ammonia/ammonium (T_{amm}) in freshwater fish (Laurén and McDonald, 1985; Wilson and Taylor, 1993; Beaumont et al., 1995; Wang et al., 1998). Both increased ammonium production and inhibited T_{amm} excretion could explain such observations. Elevated plasma cortisol, which has been reported to occur in copper exposed fish (Donaldson and Dye, 1975; De Boeck et al., 2000), stimulates protein catabolism and thus metabolic ammonia production and could be an explanation for the observed hyperammonaemia. Absolute T_{amm} excretion rates do not appear to change in response to copper exposure in rainbow trout and common carp (*Cyprius carpio*) (Laurén and McDonald, 1985; De Boeck et al., 1995). However, this unchanging T_{amm} excretion occurred in situations where plasma T_{amm} concentrations were elevated and the plasma/water gradient thus would favor higher T_{amm} excretion. The constant T_{amm} excretion rates despite a greater T_{amm} gradient could thus indicate that T_{amm} excretion is impaired. Consequently, it appears that the elevated plasma T_{amm} observed during copper exposure is the product of both elevated ammonia production and inhibited T_{amm} excretion across the gills. Although the mechanisms of T_{amm} excretion remain controversial, inhibition of the branchial Na/K-ATPase could result in reduced T_{amm} excretion or unchanged excretion in situations were the T_{amm} gradient is greatly increased due to hyperammonaemia, since ammonium can replace potassium as a substrate for the Na/K-ATPase (Mallery, 1983; Towle and Hølleland, 1987; Wall and Koger, 1994). An additional explanation for apparently reduced T_{amm} excretion could be inhibition of either intracellular or extracellular carbonic anhydrase. Acidification of the boundary water layer at the apical surface of the gill epithelium seems to be important for ammonia excretion via diffusion trapping as outlined above. Inhibition of intracellular carbonic anhydrase would deplete proton carriers in the apical membrane of substrate and thus reduce the proton excretion and thereby acidification of the boundary layer. Similarly, inhibition of extracellular carbonic anhydrase in the boundary layer would reduce the hydration of excreted carbon dioxide and thus reduce the acidification of this micro-environment. It thus seems that inhibition of branchial carbonic anhydrase could offer an explanation for inhibition of T_{amm} excretion, but this remains to be investigated.

5.1. The effect of silver on sodium and chloride homeostasis

An early study by Holm-Jensen (1948) demonstrated that silver, like copper, acts as an osmoregulatory toxicant but almost five decades passed before Wood et al. (1996a) investigated the physiological mechanism of silver toxicity in freshwater rainbow trout. These studies demonstrated that acute exposure to low silver concentrations results in similar gradual decrease in plasma sodium and chloride concentrations. The later study also revealed inhibition of branchial sodium uptake. Shortly after, Morgan et al. (1997) demonstrated that this inhibition of sodium influx was the result of non-competitive inhibition (reduced maximum transport capacity). The sodium transport affinity

was not influenced by silver exposure, demonstrating slight differences in the action of copper and silver. These findings have since been confirmed by Bury and Wood (1999). The study by Morgan et al. (1997) also demonstrated that silver induced the inhibition of the branchial Na/K-ATPase, possibly explaining the reduced sodium uptake. Inhibition of sodium influx *and* reduction in Na/K-ATPase by silver exposure have since been confirmed for rainbow trout, European eel, *Anguilla anguilla* (Grosell et al., 2000) and the freshwater crayfish, *Cambarus diogenes diogenes* (Grosell et al., 2002) and thus appears to be a general phenomenon. In contrast to copper, silver does not appear to cause increased sodium efflux across the gill epithelium in fish, although it does in crayfish (Grosell et al., 2002). As reported for copper, the interactions between silver and the Na/K-ATPase enzyme seems to be of a competitive nature at the magnesium binding site (Ferguson et al., 1996), which is critical for phosphorylation of the enzyme.

Inhibition of chloride uptake by silver exposure has been documented for rainbow trout (Morgan et al., 1997; Webb and Wood, 1998; Grosell et al., 2000), but chloride uptake in the European eel is not influenced (Grosell et al., 2000). In the studies by Morgan et al. (1997) and Webb and Wood (1998), inhibition of chloride and sodium transport occurs simultaneously, immediately after onset of silver exposure. This suggests that cytosolic carbonic anhydrase, in addition to the Na/K-ATPase, could be sensitive to silver exposure since the activity of this enzyme provides protons and bicarbonate for the apical exchange with sodium and chloride, respectively. An inhibition of this enzyme would thus result in an almost parallel inhibition of sodium and chloride influx. The study of Morgan et al. (1997) reported inhibition of branchial carbonic anhydrase, which supports the idea that carbonic anhydrase inhibition could be involved in the toxicity of silver. Precise parallel time course studies of silver-induced inhibition of sodium and chloride influx together with measurements of activity of branchial carbonic anhydrase and Na/K-ATPase are required to test this hypothesis. The apical sodium channel is another possible target for silver induced inhibition of sodium uptake since silver, like copper, interacts with sodium at the apical membrane by sharing this entry step (Bury and Wood, 1999).

5.2. Silver and T_{amm} excretion

Similar to copper, silver causes plasma hyperammoniaemia. In contrast to the situation for copper, where a combination of elevated metabolic ammonia production and inhibited ammonia excretion seems to be the case, Webb and Wood (1998) demonstrated that elevated metabolic ammonia production was the only reason for the observed increase in plasma T_{amm} in rainbow trout. This conclusion was based on observations of parallel increases in plasma T_{amm} and T_{amm} excretion rates showing that T_{amm} excretion is simply a product of T_{amm} gradient across the gill epithelium. These results have since been confirmed for a freshwater crayfish (Grosell et al., 2001) and could thus appear to be a general phenomenon.

5.3. Etiology of acute copper and silver toxicity in freshwater organisms

Although subtle differences in the toxic mechanisms of copper and silver exist, the overall etiology of copper and silver-induced mortality seems to be very similar (Wilson and Taylor, 1993; Hogstrand and Wood, 1998).

The net loss of sodium and chloride across the gill epithelium arising from the inhibition of their active uptake and possibly their increased diffusive loss sets up an osmotic imbalance between plasma and tissues. This leads to a movement of fluid from the extracellular space to the intracellular compartments reducing the plasma volume leaving plasma protein and blood cells more concentrated. In addition, during exposure to copper but not silver, splenic contraction, which causes an increased release of blood cells into circulation, and blood cell swelling increases blood viscosity (Wilson and Taylor, 1993 vs. Webb and Wood, 1998). In addition to this increased viscosity elevated cardiac output and vascular resistance as a result of catecholamine-induced systemic vasoconstriction, leads to cardiovascular collapse. This suite of physiological effects is very similar to events reported from acid-exposed rainbow trout (Milligan and Wood, 1982), in which branchial sodium and chloride transport are the primary targets for acid toxicity.

Although death after copper and silver exposure is due to cardiovascular collapse, the cascade of events leading to death starts with the inhibition of sodium and chloride uptake across the gills.

Consequently, attempts of explaining the great variability seen in sensitivity to these metals should focus on these transport mechanisms, since they are the primary target for acute toxicity. With the notable exceptions of the European eel and the American eel, *Anguilla rostrata* (Goss and Perry, 1994; Grosell et al., 2000), chloride and sodium uptake across the gill of freshwater organisms seems to be coupled. Since the mechanisms of sodium uptake are better understood and more literature exists describing sodium rather than chloride transport rates, the following discussion will focus on sodium transport.

6. Size and sodium turnover

All freshwater organisms are osmoregulators, maintaining an extracellular environment much more concentrated than the surrounding freshwater. This is especially true for sodium and chloride, resulting in continuous diffusive loss of these two ions from the animal. To maintain ion homeostasis, active absorption of these ions is necessary.

It follows from simple allometry that smaller organisms exhibit a large surface area to body mass ratio. Indeed, mass specific gill surface area increases with decreasing body mass in both fish and crustaceans (Huges and Morgan, 1973; Santos et al., 1987). Assuming that diffusive sodium loss rate is a function of surface area, the prediction would be that smaller freshwater organisms would have higher mass-specific diffusive sodium loss and that the sodium uptake rates required to maintain homeostasis would thus be higher. A review of more than six decades of reported sodium flux rates from freshwater organisms confirms this prediction. Fig. 2 presents a high number of mass-specific, sodium uptake rates as a function of body mass and clearly demonstrates that smaller organisms require a higher sodium uptake rate than larger animals to maintain internal sodium homeostasis.

With this relationship of sodium uptake rate and body mass in mind and a preliminary assumption of a constant internal set point for sodium homeostasis across all groups of freshwater animals, the expectation would be for smaller animals to have a higher sodium turnover relative to larger organisms. Note that most of the reported studies were conducted under different conditions, which undoubtedly is part of the reason for the variation which cannot be explained by body mass alone.

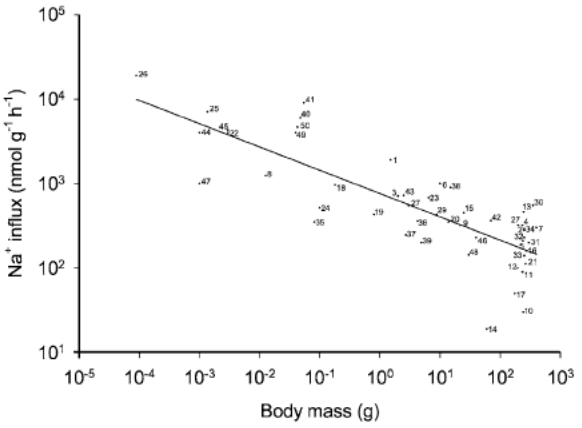

Fig. 2. Sodium uptake rates (nmol g^{-1} h^{-1}) as a function of body mass (g). The relation can be described using Eq. (7) (see text for details). Linear regression ($\log J_{in} = 2.87 - \log(w) \times 0.274$) yields $r^2 = 0.65$. Numbers refer to the following original publications: (1) Pelgrom et al. (1995); (2) Laurén and McDonald (1985); (3–4) Reid and McDonald (1988); (5) Sola et al. (1995); (6) Shaw (1959b); (7) Salama et al. (1999); (8–9, 25–26) Bianchini et al. (2002b); (10–13) Perry et al. (1992); (14–15) Grosell et al. (2000); (16–17) Goss and Perry (1994); (18–19) Grosell and Wood (2002); (20) Twitchen and Eddy (1994); (21) McDonald and Milligan (1988); (22) Holm-Jensen (1948); (23–24) Bury and Wood (1999); (27) Kirschner et al. (1973); (28) Hogstrand et al. (1996); (29) Bury et al. (1999a); (30) Wood et al. (1996a); (31) Webb and Wood (1998); (32–34) Morgan et al. (1997); (35) Brauner and Wood (2002); (36–37) Bury et al. (1999b); (38) Shaw (1959a); (39) Wood and Rogano (1986); (40–41) Fenwick et al. (1999); (42) Ehrenfeld (1974); (43) Packer and Dunson (1969); (44 and 47) Potts and Fryer (1979);(45) Stobbart et al. (1977); (46) Zetino et al. (2000); (48) Bryan (1960); (49) Shaw and Sutcliffe (1961); (50) Sutcliffe (1967).

7. Size and sensitivity to acute copper and silver exposure

As described above, depletion of extracellular sodium is the primary cause of death from copper and silver exposure in freshwater organisms. Consequently, an inhibition of the active sodium uptake mechanism by copper or silver would likely be more detrimental to smaller organisms, as sodium depletion would occur faster as a result of the higher turnover. Fig. 3 and Fig. 4 illustrate that this is the case for both copper and silver, respectively. Both figures compile a large number of reports on tolerance to the metal (as lethal concentration required to kill 50% of the population; LC_{50} value) presented as a function of body mass

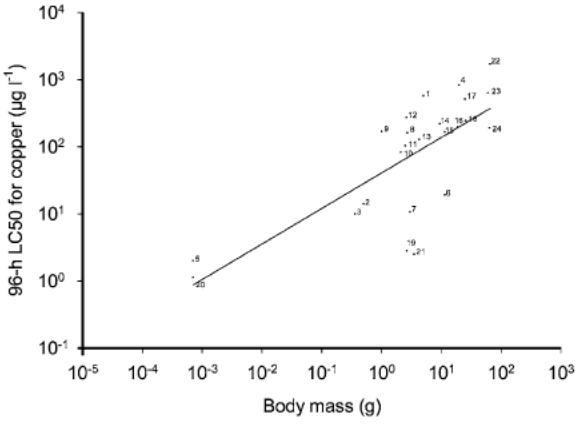

Fig. 3. Ninety-six-hour LC_{50} ($g\,l^{-1}$) for copper as a function of body mass (g). Linear regression ($\log LC_{50} = 1.61 + \log(w) \times 0.529$) yields $r^2 = 0.54$. Numbers refer to the following original publications: (1) Gill et al. (1992); (2) Marr et al. (1998); (3) Marr et al. (1999); (4) Taylor et al. (1995); (5) Welsh et al. (1993); (6) Miller and MacKay (1979); (7) Nor (1990); (8–18) Chakoumakos et al. (1979); (19) Cusimano et al. (1986); (20) Erickson et al. (1996); (21) Meyer et al. (1999); (22–24) De Boeck et al. (2001).

to reveal that smaller organisms indeed are more sensitive to both copper and silver. Note the different scale on the *y*-axis of the two figures, which illustrate that silver is a more potent toxicant than copper. Considerable variation around a still highly significant correlation in the case of both copper and silver indicates that size may be a key factor in determining relative sensitivity but also that other factors are of influence. Different water chemistries amongst the different studies is one factor of influence. Several water chemistry parameters have been documented to clearly influence the acute toxicity of both metals (see Section 1 and elsewhere in this volume). Unfortunately, many of the reported toxicity studies fail to give sufficient details concerning water chemistry in their test system. Better knowledge of water chemistry and thereby metal speciation would have allowed us to express these relationships as a function of ionic metal (Cu^{2+} or Ag^{+}, respectively) rather than as a function of total recoverable metal concentration. In addition to water chemistry, physiological factors other than body size and sodium uptake rates may also be of importance. In an attempt to evaluate the relative importance of these other physiological factors we designed a model illustrating sodium turnover in freshwater organisms.

8. Modeling sodium turnover—which parameters are important for sensitivity?

At steady state, internal sodium concentrations in aquatic animals ($[Na]_i$) are constant because sodium uptake (J_{in}) equals the diffusive sodium loss (J_{out}). Under these conditions the resulting net sodium flux is zero:

$$\frac{d[Na^+]_i}{dt} = J_{in} - J_{out} = 0 \quad (1)$$

For the modeling we have assumed a constant loss (J_{out}) regardless of metal exposure and investigate the effects of simply changing the sodium uptake (J_{in}). We characterize a change in uptake by the inhibition parameter α:

$$\frac{d[Na^+]_i}{dt} = (1-\alpha)J_{in} - J_{out} \quad (2)$$

Under normal conditions, when $\alpha = 0$, there is no inhibition of sodium uptake and when $\alpha = 1$, there is full inhibition of sodium uptake. At equilibrium (unperturbed conditions) sodium loss is balanced by sodium uptake (J^0_{out} is defined as J_{out} prior to exposure).

$$J_{in} = J_{out} = J^0_{out} \quad (3)$$

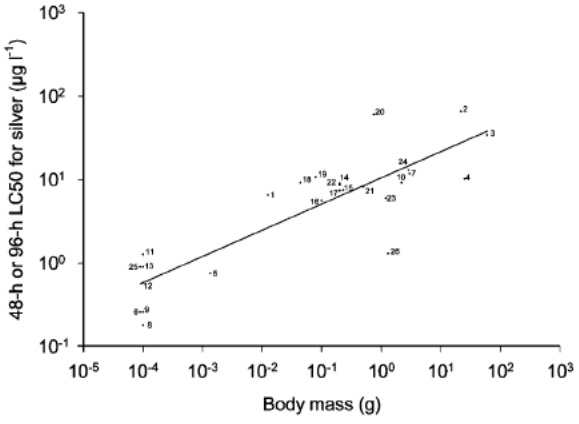

Fig. 4. Forty-eight-hour or 96-h LC_{50} ($g\,l^{-1}$) for silver as a function of body mass (g). Linear regression ($\log LC_{50} = 1.02 + \log(w) \times 0.315$) yields $r^2 = 0.75$. Numbers refer to the following original publications: (1–2 and 5–6) Bianchini et al. (2002b); (3–4) Grosell et al. (2000); (7) Hogstrand et al. (1996); (8–9) Bianchini et al. (2002a); (10) Karen et al. (1999); (11) Erickson et al. (1998); (12–14 and 17–18) Nebeker et al. (1983); (15–16) Bury et al. (1999a); (19–20) Lima et al. (1982); (21) Buccafusco et al. (1981); (22) Norbert-King (1989); (23–27) Holcombe et al. (1982).

As the variation in J_{in} is given by $(1-\alpha)$, the resulting net sodium flux $((1-\alpha)J_{in}-J_{out})$ can be expressed simply as $\alpha(J_{out})$.

For a given body weight, we assume a constant initial internal sodium concentration and constant sodium loss per unit time. This implies that the internal sodium concentration decays exponentially if sodium uptake is inhibited (t=time):

$$[Na]_i(t)=[Na]_i(0)\exp(-t/\tau) \quad (4)$$

where the time constant τ is given by:

$$\tau=\frac{[Na]_i(0)}{\alpha J^0_{out}} \quad (5)$$

Mortality during acute exposure to elevated levels of copper and silver occurs when plasma sodium concentrations are reduced by approximately 30% (Wilson and Taylor, 1993; Wood et al., 1996a). Consequently time to death due to inhibition of sodium uptake ($T_{70\%}$) will be:

$$T_{70\%}=-\tau\ln(0.7)=\frac{[Na]_i(0)\ln(0.7)}{\alpha J^0_{out}} \quad (6)$$

Fig. 2, shows the relation between sodium uptake and body mass. The relation can be described by a power law (see figure legend):

$$J^0_{out}=10^{287}w^{-0\,274} \quad (7)$$

where w is the body weight in grams. The time constant will thus depend on body weight. Combining Eq. (5) and Eq. (7) we obtain:

$$\tau=\frac{[Na]_i(0)}{\alpha J^0_{out}}=\frac{[Na]_i(0)}{\alpha\times10^{2\,84}w^{-0\,274}} \quad (8)$$

Substituting Eq. (8) into Eq. (6) we can express the time to mortality ($T_{70\%}$) as:

$$T_{70\%}=\frac{-\ln(0.7)[Na]_i(0)}{\alpha\times10^{2\,84}w^{-0\,274}} \quad (9)$$

The derivation of Eq. (9) is based on the assumption that all internal sodium is available for exchange. However, in fish, only 30% of the body mass (extracellular fluid, referred to as p in the following) is available for sodium exchange with the surrounding environment (Wood and Randall, 1973). Thus the actual time to mortality will be proportional to the fraction p of total body sodium:

$$T_{70\%}=\frac{-\ln(0.7)[Na]_i(0)p}{\alpha\times10^{2\,84}w^{-0\,274}} \quad (10)$$

As an example, a typical 100 g freshwater fish will have a sodium uptake rate of 196 nmol g^{-1} h^{-1} according to the relation between body mass and sodium uptake shown in Fig. 2 and for a typical freshwater fish, the extracellular sodium concentration is approximately 150 mM. Assuming a metal induced inhibition of branchial sodium uptake of close to 100%, Eq. (10) predicts that a level of 30% reduction in plasma sodium (and thus mortality) for a 100 g fish will be reached after 82 h or approximately after 3 days of exposure.

From Eq. (10), it clearly follows that mortality will occur later for animals with higher internal sodium concentration, larger relative exchangeable sodium volume, and lower sensitivity to depletion of internal sodium. Animal mortality is also sensitive to sodium turnover but the situation is more complex. Both sodium uptake and body mass influence sodium turnover. For animals with comparable sodium uptake rates but different body mass, Fig. 2 and Eq. (7) predicts that a larger animal will experience depletion of internal sodium pools at a lower rate than a smaller animal. Conversely, for animals of comparable size but different sodium uptake rates, animals with higher sodium uptake rates will experience depletion of internal sodium pools at a higher rate than animals with lower sodium uptake rates. Nevertheless, mortality will occur earlier in animals with higher relative inhibition of sodium uptake.

9. Importance of individual parameters for predicting sensitivity to copper and silver

The exchangeable sodium pool is the product of accessible sodium volume and concentration. Therefore, intracellular sodium is of little importance due to the low concentration in this compartment. In contrast sodium concentration in the extracellular compartments is high. For example larger decapods like crayfish can have hemolymphic sodium concentrations of approximately 190 mM (Grosell et al., 2002) and rainbow trout sodium concentrations are approximately 150 mM (Wood et al., 1996a,b). In general, aquatic freshwater animals exhibit limited variation in the extracellular sodium concentration and the 20% difference in extracellular sodium between crayfish and rainbow trout is not sufficient to explain the big difference (>5-fold) in silver tolerance (Bianchini et al., 2002b).

Extracellular fluid volume in crustaceans and fish is very similar (Prosser and Weinstein, 1950; Nicholas, 1987; Steffensen and Lomholt, 1992) even though blood volume varies considerably between crustaceans with open circulation (25% of body mass) and teleost fish with closed circulation (2–4%). This similarity arises because in teleosts the exchangeable sodium volume is equal to the extracellular fluid volume (plasma volume + interstitial fluid volume) as a consequence of the rapid exchange between plasma and interstitial fluid (Nicholas, 1987). The implication is that variations in exchangeable sodium volume amongst freshwater organisms (i.e. crustaceans and fish) will have negligible influence on copper and silver tolerance within the time frame typically considered in toxicity testing.

This raises the question, how is the threshold for metal-induced mortality in crustaceans and fish is related to the degree of plasma sodium depletion? We note that for both copper and silver, a 30% reduction in rainbow trout plasma sodium results in mortality, but the threshold for metal-induced sodium depletion for crustaceans is unknown.

In a recent study of the physiology of silver toxicity in adult crayfish, most animals survived a 30% reduction in hemolymph sodium concentration (Grosell et al., 2002) at 8.4 g silver l^{-1} (as silver nitrate), which is considerably lower than the LC_{50} values reported for the same species under similar conditions (Bianchini et al., 2002b). This may suggest that crayfish or possibly crustaceans have a higher tolerance to reduced hemolymph sodium concentrations than teleost fish but it remains to be investigated.

Unlike the parameters discussed above, both body mass and sodium uptake/loss rates vary by several orders of magnitude amongst different freshwater organisms. Therefore body mass and sodium uptake/loss will be important in determining the relative sensitivity to copper and silver. Body mass and sodium uptake/loss rates are correlated as illustrated in Fig. 2 although with considerable variation amongst different species of comparable size. A striking example is the comparison of the European eel and the rainbow trout which exhibit a 20-fold difference in sodium uptake rates at comparable body mass (Grosell et al., 2000). The eel has the lowest sodium uptake rate and also the lowest sensitivity to acute silver exposure.

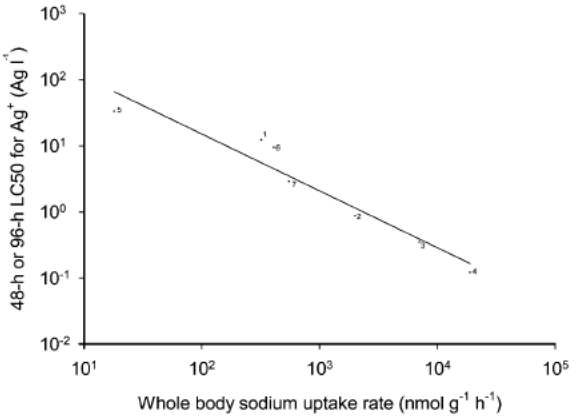

Fig. 5. Forty-eight-hour or 96-h LC_{50} ($g\,l^{-1}$) for ionic silver (Ag^+) calculated by MINEQL$^+$ software. Linear regression ($\log LC_{50} = 2.89 - \log(J_{in}) \times 0.860$) yields $r^2 = 0.93$. Numbers refer to the following original publications: (1–4) Bianchini et al. (2002b); (5) Hogstrand et al. (1996); (6–7) Grosell et al. (2000).

10. Applications of the sodium turnover model

For small animals (i.e. daphnids, 10–100 μg) bioassays typically last less than 48 h whereas for larger animals (i.e. fish and crayfish, 10–100 g) bioassays typically last 96 h. In Fig. 4 and Fig. 5, toxicity data from 48- and 96-h bioassays are included in the same regression analysis. This may seem problematic, but for small animals mortality occurs before 48 h of exposure (Bianchini et al., 2002b). The sodium turnover model predicts mortality for animals of 100 g body mass at less than 35 h with 5% inhibition of sodium uptake. In comparison, for large animals, the sodium turnover model predicts that depletion of internal sodium will take longer, i.e. that mortality will occur later (e.g. at 82 h for a 100 g animal, see above). The model predictions suggest that it is reasonable to compare LC_{50} values obtained from 48-h bioassays on small animals with LC_{50} values obtained from 96-h bioassays on larger animals. The sodium turnover model thus becomes useful in the planning and design of bioassays. To demonstrate this, let us first consider a large (>100 g) animal with normal sodium transport rates. For a typical 300-g animal mortality would occur after 100 h thus exceeding the standard 96-h bioassay time frame. This suggests that a 96-h bioassay may not be sufficient to determine a reliable LC_{50} value for large animals. Let us now consider a 100-g animal with an low sodium uptake/loss rate of 40

nmol g^{-1} h^{-1}, which is typical for the eel species (*Anguilla* sp.) (Fig. 2). For this animal mortality would occur after 401 h again exceeding the standard 96-h bioassay time frame.

The present model offers an explanation for the high sensitivity of smaller freshwater invertebrates. As outlined above, a 5% inhibition of sodium uptake in a 100-μg animal would lead to mortality in less than 35 h, whereas a much more complete inhibition (close to 100%) is needed to induce mortality in a 100 g organism within 82 h. These predictions are based on the assumptions that fish and small freshwater invertebrates have similar mechanisms for sodium uptake and for acute silver and copper toxicity. These assumptions seem valid when comparing the toxic mechanism of acute silver exposure in the larger invertebrate, the crayfish to the fish (Grosell et al., 2002), but they remain to be verified for smaller invertebrates. Under the above assumptions we would expect that all freshwater organisms exhibit an increased inhibition of sodium uptake with increasing metal concentration. The high sensitivity of small freshwater animals to low metal concentrations could be explained by noting that a very limited inhibition ($<5\%$) of sodium uptake is necessary to induce mortality within a standard 96-h bioassay. In contrast to smaller animals, almost complete inhibition ($>95\%$), i.e. high metal concentrations, are needed to induce mortality in larger animals within a 96-h bioassay.

11. Limitations of the model

The present model gives an estimate of relative sensitivity of a given organism simply based on size. Such an estimate is associated with considerable uncertainty because sodium flux rates vary significantly amongst animal species of similar size. If the sodium uptake rate of a given organism is known, the model prediction will be improved. The amount of data for sodium uptake in freshwater organisms is considerably larger than toxicity data for both copper and silver combined. This implies that sodium uptake data can be utilized to identify species particularly sensitive to metal exposure and thus predict time to mortality. To predict relative sensitivity to metal exposure from sodium uptake rates, one can simply replace Eq. (7) with the measured absolute sodium uptake rate from the organisms in question.

One of the underlying assumptions for the model is that J_{out} is not altered during metal exposure, which is not always the case. As previously mentioned, copper exposure at higher concentrations increase J_{out} at least in rainbow trout. Furthermore, crayfish exhibit a transient increase J_{out} during acute silver exposure (Grosell et al., 2002). In addition, acclimation responses during exposure have also been reported. These acclimation responses include restored sodium uptake even during continued copper exposure (Laurén and McDonald, 1987a,b), and also reduced renal sodium loss to compensate for the impaired sodium uptake in rainbow trout (Grosell et al., 1998). The present model does not account for metal-induced increases in J_{out} or acclimation responses and the model should only be used in predicting the effects of acute response to environmentally realistic concentrations of metals.

12. Silver sensitivity and sodium flux

The applicability of the BLM geochemical model which is based on teleost fish and extended to account for smaller freshwater invertebrates is limited by the fact that sodium turnover rates are not considered. We have investigated the combined effects of metal speciation and the effect of sodium uptake rates on metal sensitivity and this is shown in Fig. 5.

Fig. 5 shows LC_{50} silver concentration (as ionic silver using MINEQL$^+$ software) as a function of sodium uptake rate. When sodium uptake rate is combined with metal speciation we obtain a close correlation between tolerance and sodium turnover consistent with earlier findings (Bianchini et al., 2002b). This implies that both sodium uptake rate and metal speciation should be considered when attempting to predict tolerance to silver exposure. The analysis in Fig. 5 is contingent on sufficiently detailed information about the water chemistry. Unfortunately, this information is limited for copper exposure studies, and a similar plot for copper could not be constructed.

13. Conclusions

There is a striking similarity between the action of copper and silver on gill physiology although differences exist between the mechanisms of acute toxicity of these metals. Geochemical speciation models combined with the chemical characteristics

of the gill (the biotic ligand) have enhanced our understanding of variations in sensitivity to both metals within a single group of aquatic organisms. However, these models cannot explain the large observed variation in metal sensitivity amongst different groups of freshwater animals. The present review demonstrates that the observed variations amongst freshwater animals can be attributed to variations in sodium uptake/loss rates as illustrated for silver in Fig. 5. Furthermore, as sodium uptake rates are correlated with body mass one can estimate relative metal sensitivity of a given organism directly from body mass.

14. Recommendations

Realizing that the ionic form of the metal is critical for toxic responses, detailed information about water chemistry should always be included in reports of toxicity testing.

While animal size (weight) is often reported in toxicity studies, it is not always the case. In some studies no information is given, while others refer length (of fish larvae) or simply age of the animals tested. Weighing very small organisms (in the microgram range) can present a practical problem, however, the average weight of a group of animals provide at least a mean value and should always be included. Reviewing the literature revealed that several studies used a wide range of animal sizes (an example could be 1–5 g). This difference in body mass alone would introduce a 57% variation in time to internal sodium depletion and thus time to death according to our model. The consequence of using animals of variable size results in additional variation in the data set, which may mask correlations. If variable size is unavoidable, great care should be taken to randomize distribution of animal sizes between experimental treatments to avoid artifacts.

As discussed in detail above, carbonic anhydrase may be an important target for both copper and silver toxicity in freshwater animals. This potential target has so far been underestimated. Fine grained time course studies of parallel sodium and chloride transport in combination with measurements of carbonic anhydrase and Na/K-ATPase activity are needed to evaluate the importance of a possible inhibition of carbonic anhydrase mediated transport processes.

Studies of acute silver and copper toxicity in smaller freshwater invertebrates should include measurements of tolerance to reduction in hemolymph sodium concentrations as well as measurements of unidirectional ion fluxes in crustaceans.

Since copper and silver apparently share targets in/on the gill epithelium, possible interactions between the two metals are likely to occur during a mixed exposure. Emissions of both metals through sewage treatments plants means that mixed exposure may well occur in natural environments. An evaluation of potential synergistic and antagonistic interactions between these two metals is clearly needed.

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