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Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates

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Abstract

The present paper presents original data and a review of the copper (Cu) toxicity literature for estuarine and marine environments. For the first time, acute Cu toxicity across the full salinity range was determined. Killifish, *Fundulus heteroclitus*, eggs were hatched in freshwater (FW), 2.5, 5, 10, 15, 22 and 35 ppt (seawater, SW) and juveniles were allowed to acclimate for 7 days prior to acute toxicity testing. Sensitivity was highest in FW (96 h LC50: 18 μg/l), followed by SW (96 h LC50: 294 μg/l) with fish at intermediate salinities being the most tolerant (96 h LC50 > 963 μg/l at 10 ppt). This approximately 50-fold, non-linear variation in sensitivity could not be accounted for by Cu speciation or competition among cations but can be explained by physiology. The relative Na^+ gradient from the blood plasma to the water is greatest in FW followed by SW and is smallest at 10 ppt. Regression of Cu toxicity versus the equilibrium potential for Na^+, which reflects the relative Na^+ gradient, revealed that 93% of the variation can be attributed to Na^+ gradients and thus osmoregulatory physiology. Examination of the existing literature on acute Cu toxicity in SW (defined as >25 ppt) confirmed that early life stages generally are most sensitive but this pattern may be attributable to size rather than developmental stage. Regardless of developmental stage and phylogeny, size clearly matters for Cu sensitivity. The existing literature on the influence of salinity on acute Cu toxicity as well as studies of mechanisms of Cu toxicity in fish and invertebrates are reviewed.

Keywords: Killifish; *Fundulus heteroclitus*; Toxic mechanism; Cu; BLM

1. Introduction

Cu is an essential micronutrient and acts as a co-factor in multiple enzymatic processes but is potentially toxic to aquatic organisms. While Cu is present in all aquatic environments, multiple anthropogenic activities may result in elevated concentrations, increased exposure, and potential toxicity to aquatic organisms. However, the concentration of Cu in the aquatic environment is not the sole factor dictating the likelihood of adverse effects. A wealth of studies has demonstrated unequivocally that complexation of Cu with organic and inorganic ligands as well as competition with other cations for binding and uptake pathways greatly influence Cu toxicity in FW (McGeer et al., 2002; Paquin et al., 2002; Santore et al., 2001). This strong influence of environmental parameters on Cu toxicity to FW organisms is recognized by the USEPA and has been incorporated into a Biotic Ligand Model (BLM) that is currently employed to allow for the establishment of site specific water quality criteria (USEPA, 2003). The FW BLM uses water chemistry to predict the amount of Cu which will accumulate on/in the gill of a FW organism and estimates toxicity from a relationship between gill Cu accumulation and mortality. The gill was chosen as the target organ for BLM modeling because multiple studies demonstrate that this organ rapidly accumulates Cu following the onset of waterborne exposure and this results in the disturbance of multiple physiological processes.

While disruption respiration in FW organisms may be the cause of mortality at high Cu concentrations, mortality as a result of environmentally relevant Cu concentrations is most often associated with osmoregulatory disturbance (Grosell et al., 2002).

All FW organisms maintain internal salt concentrations above those of the surrounding environment and fall in the category of osmoregulators. They also all must regulate internal acid–base balance and excrete nitrogenous waste. The commonality of all FW organisms relying on active uptake of Na^+ combined with

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the specific action of Cu on Na^+ uptake pathways (Grosell et al., 2002; Grosell and Wood, 2002) explain why the FW BLM is successful in predicting acute toxicity in both fish and invertebrates. However, it should be noted that although the BLM accounts for much of the variation in Cu toxicity associated with water chemistry, considerable variation in sensitivity exists among species. An examination of available data for Cu sensitivity in FW organisms revealed that the majority of this among-species variation could be accounted for by differences in size or rather differences in rates of Na^+ turnover (Grosell et al., 2002). A strong correlation exists between Na^+ turnover rates and size in FW organisms (Grosell et al., 2002). Currently the strong influence of size is not recognized by the BLM and species differences in sensitivity are simply dealt with through adjustments of constants characterizing the metal/gill interaction. In this regard, it should be realized that reported acute Cu toxicity values (as LC50's) span three orders of magnitude. Water chemistry alone certainly can influence acute toxicity to a single species by as much as one order of magnitude but this leaves the majority of the overall variation unaccounted for. A large portion of the remaining variation is due to differences in size and thus difference in physiology among tested species (Grosell et al., 2002).

The large variation among species (>3 orders of magnitude) presents a challenge for the establishment of sensible environmental regulation of Cu since it will be impossible to test all organisms, including endangered species, and thus capture the full range of sensitivity. Considering the wide range in sensitivity for a relatively small number of species tested the most sensitive (and tolerant) species likely have yet to be identified. This challenge is best met by understanding the sources of the variation in sensitivity within and among species to allow for extrapolation to organisms for which data cannot be obtained. Such an understanding must include an understanding of the physiological basis for variation in sensitivity, a point which becomes even more important when considering Cu toxicity in marine and estuarine environments.

Currently, there is considerable effort being made towards the development of a BLM for marine environments and environments of intermediate salinity. Similar to FW environments, organic and inorganic complexation of metals are anticipated to be major factors influencing bioavailability and toxicity. A number of studies have demonstrated the importance of DOC in marine systems (Arnold, 2005; Lorenzo et al., 2002; Sunda et al., 1987) and inorganic complexation of some metals, such as cadmium by chloride, have also been shown to be important (Lin and Dunson, 1993; Roast et al., 2001; Wildgust and Jones, 1998). However, for other metals such as Cu, inorganic complexation does not appear to be of great importance in defining toxicity (Blanchard and Grosell, 2006). Despite this, differences in inorganic water chemistry associated with increasing salinity may tend to reduce Cu toxicity as illustrated by studies on both invertebrates and fish (Fernandez and Jones, 1990; Jones et al., 1976; Olson and Harrel, 1973; Reardon and Harrell, 1990; Verslycke et al., 2003).

Additionally, the available data for acute Cu toxicity in SW is no less variable than in FW (Fig. 1). The species sensitivity diagram (SSD) provided in Fig. 1 illustrates a variation of around four orders of magnitude among species/studies in acute toxicity (see figure legend for details). Data collected from salinities of 25–38 ppt are included in Fig. 1 and water chemistries clearly varied among the studies forming the basis for the SSD. However, the variations in water chemistry at these high salinities are relatively small compared to relative variations in FW chemistry and therefore likely explain only a modest fraction of the variation in sensitivity.

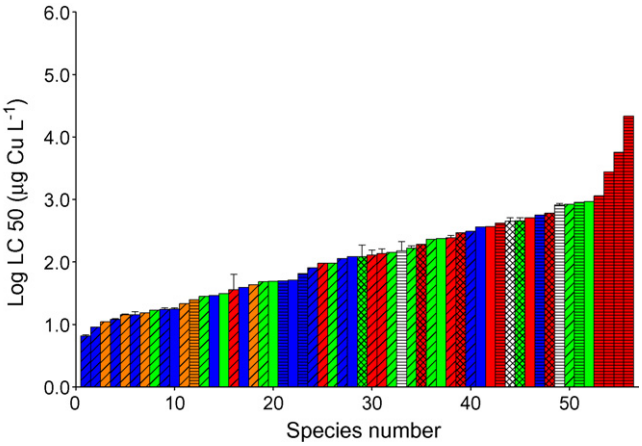

Fig. 1. Species sensitivity distribution of organisms exposed to copper in seawater. Life stages are represented by the hatching: single cross hatch are embryos, larvae, and eggs, double cross hatch are juveniles, and horizontal hatch are adults. Data were obtained from (USEPA, 2003) Table 1b (species #1, 4, 5, 8–11, 14–17, 19–23, 25–38, 40, 42–49 and 51–55). Tests performed in 25 ppt and greater were included and the LC 50's for common life stages were averaged with error bars indicating SEM. Species #53 (sheepshead, *Archosargus probatocephalus*), #54 (pinfish, *Langodon rhomboides*), and #55 (Atlantic croaker, *Micropogon undulatus*) were considered to be adult based on the reported size. Additional data were obtained for grooved carpet snail, *Ruditapes decussates*, embryos, #2 (Beiras and Albentosa, 2004), sea urchin, *Diadema antilarum*, larvae, #3 (Bielmyer et al., 2005), Meditteranean mussel, *Mytilus galloprovincialis*, embryos, #6 (Arnold et al., 2006), purple sea urchin, *Stongylocentrotus purpuratus*, #7 (Phillips et al., 2003), sea urchin, *D. antilarum*, adults, #12 (Bielmyer et al., 2005), *Artemia franciscana* cysts, #13 (Brix et al., 2006), reef sea urchin, *Diadema setosum*, embryos, #18 (Ramachandran et al., 1997), Phillipines oyster, *Crassostrea iradalei*, embryos, #24 (Ramachandran et al., 1997), *Nassarius festivus*, #41 (Cheung et al., 2002), northern pink shrimp, *Peneaus duorarum*, Larvae, #50 (Cripe, 1994), killifish, *Fundulus heteroclitus*, juveniles (species #39, present study), and toadfish, *Opsanus beta* #56 (Grosell et al., 2004b).

We present the argument that the majority of the variation among studies included in Fig. 1 can be attributed to differences in physiology rather than water chemistry (which presumably varied little among these studies performed at high salinities). In an attempt to further investigate this argument, we have examined the data for the influence of phylogeny, developmental stage, and body mass on sensitivity to acute Cu exposure.

In contrast to the situation in FW where all animals are osmoregulators, multiple strategies are employed for the control of salt and water balance in SW organisms. All teleost fish regulate extracellular ionic concentrations much below that of SW and are thus both osmo- and iono-regulators. All elasmobranch fish maintain osmotic pressure of extracellular fluids similar to the surroundings but maintain ion concentrations at approximately 50% of SW (the discrepancy made up by organic osmolytes) and are thus characterized as osmoconformers

and iono-regulators (Grosell, 2006). Most marine invertebrates employ a third strategy of osmo- and iono-conformity in which ionic composition and osmotic pressure greatly resemble that of the marine environment. Considering that Cu targets ion transport (Grosell et al., 2002, 2004a,b) as well as acid–base transport (Wang et al., 1998; Wilson and Taylor, 1993a) and ammonia excretion (Blanchard and Grosell, 2006; Grosell et al., 2003; Wilson and Taylor, 1993a) which ultimately rely on Na^+ gradients (and Cl^- gradients for HCO_3^- transport), it seems plausible that differences in strategies for maintaining salt balance may explain some of the variation in sensitivity to Cu.

There is little doubt that the basis for variation in acute Cu toxicity in SW is more complicated than in FW and that SW organism physiology (which is diverse) rather than chemistry (which is relatively stable) would dominate the variation. However, the degree of complexity likely increases further when we consider intermediate salinities where both chemistry and physiological strategies are more diverse. The understanding of how differences in physiology among distinct phylogenic groups may influence sensitivity to Cu at different salinities is currently hindered by the limited number of studies evaluating the influence of salinity on Cu toxicity. A few studies form exceptions and provide some insight (Blanchard and Grosell, 2005; Blanchard and Grosell, 2006; Fernandez and Jones, 1990; Jones et al., 1976; Olson and Harrel, 1973; Reardon and Harrell, 1990; Verslycke et al., 2003), but no studies to date have examined the influence of salinity on acute toxicity across the full range of salinities.

Consequently, one aim of the present study was to determine the 96 h LC50 for Cu in a fully euryhaline fish, the killifish (*Fundulus heteroclitus*), across the full range of salinities and further to attempt to explain the expected differences in sensitivity by water chemistry and physiology. An additional aim of the present report is to review the current information available regarding mechanisms of toxicity in fish and invertebrates in SW and at intermediate salinities and to look for unifying trends in the data available. Finally, based on our experimental data and the literature review we provide suggested directions for future research in this challenging and exciting area.

2. Materials and methods

2.1. Experimental animals

Larval killifish (*F. heteroclitus*) were produced from an in-house culture of wild caught specimens (St. Augustine, FL). Adult killifish were maintained in flow-through SW at ambient temperature and were fed *ad libitum* commercially available dry pellet food. Groups consisting of one male and three to four females were placed in separate tanks. For spawning, tanks were fitted with egg laying substrates overnight. Egg laying substrates consisted of short (2 cm) lengths of PVC tubing covered with fine plastic mesh in the bottom and a wider plastic mesh on the top. The wider plastic mesh on the top of the PVC tubing allowed for eggs to fall through and collect on the fine mesh in the bottom while preventing the adults from having access to the eggs. Each morning eggs were removed from the spawning tanks, rinsed briefly in de-ionized water and were placed on moist (FW) papers towels in plastic petri-dishes. The covered petri-dishes were placed in an environmental chamber at 25 °C and fungus infected eggs and unfertilized eggs were removed daily. At day 14 post-fertilization, eggs were placed in 1 l Tripour® plastic beakers containing 0.8 l of water at the planned test salinity (a total of seven salinities ranging from FW to SW). The water was vigorously aerated until eggs hatched (typically within 10 min of immersion) after which aeration was reduced and newly hatched *Artemia* nauplii were added. Hatching success of eggs that had developed through 14 days of incubation was >90% regardless of salinity and larvae were fed *Artemia* nauplii *ad libitum* daily. All toxicity tests were initiated with 7-day-old fish.

2.2. Bioassays

At 7 days of age, bioassays were performed in FW, 2.5, 5.0, 10.0, 15.0, 22.0 and 35 ppt (SW). Juvenile fish were exposed to Cu (as $CuSO_4$) in the salinity at which they were hatched. The FW used was 2:1 dechlorinated city of Miami tap water:de-ionized water while SW (from Bear Cut between Virginia Key and Key Biscayne, Florida) was used as the highest salinity. The 2:1 dilution of the Miami city tap water was chosen to keep DOC concentrations in the freshwater, seawater and thus intermediate salinities the same. All intermediate salinities were made from mixes of these two media and measured water chemistry is provided in Table 1.

Toxicity tests (96 h) were performed at 25 °C in triplicate in 1 l plastic Tripour® beakers (USEPA, 1993). Test solutions were prepared 24 h in advance to allow for stabilization of water chemistry including potential adsorption/complexation. All test solutions were replaced at 48 h (new media was also pre-equilibrated for 24 h), mortality was recorded daily and dead fish were removed from the test beakers. Prior to renewal of test solutions at 48 h, test organisms were supplied with *Artemia* nauplii and were allowed to feed for 30 min. The feeding at 48 h was included in the test procedures because it reduced control mortality especially in the lower salinities. Water samples were obtained at 48 and 96 h, passed through a 0.45 μm syringe filters (Acrodisc syringe filter; Pall Life Sciences, Houston, TX, USA) and acidified using concentrated nitric acid (trace metal grade, 1% (v/v); Fisher Scientific, Pittsburg, PA, USA).

2.3. Analytical techniques, calculations and statistical evaluation

Water from all tests was analyzed for major constituents. Cations (K^+, Na^+, Mg^{2+} and Ca^{2+}) were analyzed using a Varian FS220 flame atomic absorption spectrophotometer (Varian, Mulgrave, Victoria, Australia) while Cl^- and SO_4^{2-} were analyzed using anion chromatography (DIONEX DX 120 fitted with an AS40 automated sampler; Dionex Corp., Sunnyvale, CA, USA). Total CO_2 was measured using a total CO_2 analyzer (965 Corning limited, Halstead, Essex, England) and pH was measured using a combination glass electrode (pHc3005-8 Radiometer, Villeurbanne, Cedex, France) coupled to a meter (PHM220 MeterlabTM, Radiometer, Villeurbanne, CEDEX, France). Concentrations of HCO_3^- and CO_3^{2-} were estimated

Table 1
Water chemistry from bioassay experiments

Salinity (ppt)	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}	Total CO_2	pH	HCO_3^- [a]	CO_3^{2-} [a]	DOC [b]
FW	1.1	0.04	0.12	0.31	1.03	0.11	0.71	7.69	0.68	0.01	199.1
2.5	27.1	0.6	3.0	0.7	32.8	3.5	0.66	7.67	0.63	0.01	208.1
5	56.6	1.3	6.1	1.0	72.6	3.2	0.78	7.72	0.75	0.02	194.2
10	117.0	2.7	12.6	1.1	154.5	7.0	1.00	7.74	0.96	0.03	199.6
15	192.9	4.3	20.2	1.4	252.4	11.0	1.31	7.88	1.23	0.06	215.7
22	325.2	6.9	31.5	8.4	384.4	18.5	1.77	7.96	1.64	0.12	203.2
35	415.2	10.8	58.6	12.6	518.8	21.7	2.32	8.07	2.00	0.30	192.3

Measured water chemistry in mM.
[a] Calculated from total CO_2 and pH.
[b] As μM carbon.

from the measured pH and total CO_2 using CO2Sys software (Lewis, 1996). The concentration of dissolved organic carbon (DOC) was determined in all test solutions by high-temperature catalytic oxidation using a Shimadzu total organic carbon-VCSH (V series, combustion catalytic oxidation/nondispersive infrared method, Kyoto, Japan) (Hansell and Carlson, 2001). Concentrations of dissolved Cu in test solutions were determined by graphite furnace atomic absorption (model 220Z graphite furnace atomic absorption spectrophotometer, Varian, Mulgrave, Victoria, Australia). Matrix interference at higher salinity were overcome by solvent extraction as described elsewhere (Blanchard and Grosell, 2006).

The toxicity data were analyzed by probit analysis using the software program ToxCalc, v 5.0 (Tidepool Scientific Software, McKinleyville, CA) to estimate the LC50. Inorganic Cu speciation was calculated from water chemistry using a metal speciation program designed to handle high- and low-salinity solutions (Millero and Pierrot, 1998).

3. Results

Control survival was good in all bioassays regardless of salinity (100% in FW, 2.5 and 10 ppt and 85 ± 11, 90 ± 6, 86 ± 10 and 95 ± 5% in 5, 15, 22 ppt and SW, respectively) attesting to the euryhalinity of this species. For two (2.5 and 15 ppt) of the seven bioassays, data did not allow for calculation of confidence intervals. For one test (10 ppt) sufficient mortality could not be achieved within Cu solubility limits to estimate an LC50. The maximal dissolved Cu concentration at 10 ppt was 973 μg/l and the highest mortality was 33 ± 3%. The data from this test is presented as >973 μg/l in Figs. 1 and 2. Calculated LC50's and confidence intervals (when available) display a biphasic pattern with increasing Cu tolerance from FW to 10 ppt and reduced Cu tolerance with salinity increasing from 15 ppt to SW (Fig. 2A).

The concentration of DOC was constant across salinities, the H^+ concentration decreased with increasing salinity while all other electrolytes increased (Table 1). As salinity increased, so did pH and total CO_2, resulting in an increased fraction of Cu in the form of Cu dicarbonate. At 35 ppt, Cu carbonate and Cu dicarbonate combined to 87% of the total Cu (Table 2). The two forms of Cu generally believed to be most toxic, Cu^{2+} and $CuOH^+$, increased from FW to 2.5 ppt but then decreased with increasing salinity resulting in the lowest fractions in SW (Table 2).

4. Discussion

4.1. Differences in Cu toxicity to killifish across salinities

As expected, salinity greatly influenced acute Cu toxicity and resulted in an at least 50-fold difference between the highest and lowest LC50. At the salinity with the highest LC50 (10 ppt), 50% mortality could not be achieved due to solubility limitations meaning that the true difference in acute Cu toxicity exceeded

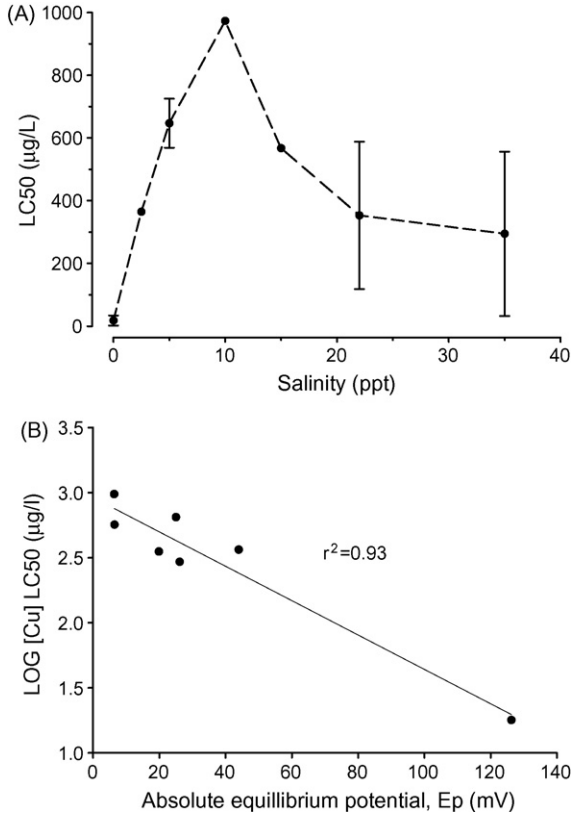

Fig. 2. Acute Cu toxicity to juvenile killifish, *Fundulus heteroclitus* (A) as 96 h LC50's as a function of ambient salinity (mean ± 95% confidence interval) and (B) as LOG 96 h LC50's as a function of the Na^+ equilibrium potential (see text for details) at different salinities.

Table 2
Cu speciation

	FW	2.5 ppt	5 ppt	10 ppt	15 ppt	22 ppt	SW (35 ppt)
Cu^{2+}	11.6	18.0	16.8	15.5	10.6	8.0	5.3
$CuOH^{+}$	5.2	6.2	5.9	5.4	5.1	4.4	3.6
$Cu(OH)_2$	1.4	1.7	1.7	1.6	2.0	1.8	1.8
$CuHCO_3^{+}$	0.4	0.3	0.3	0.3	0.2	0.2	0.1
$CuCO_3$	80.1	65.8	69.7	69.6	72.2	71.2	68.9
$Cu(CO_3)_2$	0.7	1.00	1.7	3.0	6.5	11.6	18.6
$CuSO_4$	0.5	7.0	3.9	4.6	3.5	2.9	1.8

Relative inorganic speciation of copper (% of total) across salinities used in the present study. Inorganic speciation calculations were performed based on measured water chemistry (Table 1) and measured dissolved copper (the calculated LC50 concentration) using a metal speciation program designed to handle high as well as low salinity solutions (Millero and Pierrot, 1998).

50-fold. It should be noted that the concentration of DOC was kept constant across salinities to limit the extent to which this otherwise important parameter for Cu toxicity might contribute to the observed variation in tolerance across salinities.

An expectation of increased Cu tolerance with increasing salinity seems reasonable considering that competing cations (Na^+) (Grosell and Wood, 2002) and cations known to protect against toxicity (Ca^{2+}) (Lauren and McDonald, 1985; Lauren and McDonald, 1986) are most abundant with increasing salinity (Table 1). However, the bioassay data, did not agree with this expectation and showed highest tolerance at intermediate salinities and highest sensitivity at the two extreme salinities. Speciation calculations fail to explain this pattern because the ionic Cu^{2+} and $CuOH^+$, which are generally accepted to be the toxic forms of Cu (Paquin et al., 2002), all are most abundant at the intermediate salinities. Thus it appears that Cu speciation and competition fail to account for the variations in sensitivity to Cu across salinity. Note that even though confidence interval could not be calculated for some bioassays, preventing direct statistical comparisons between individual salinities, the regression analysis presented in Fig. 2B, revealed a highly significant correlation between sodium gradients and acute copper toxicity.

The interference of Cu with Na^+ uptake pathways in FW organisms was first demonstrated almost six decades ago (Holm-Jensen, 1948) and is now well established. The reduced ability to acquire Na^+ from FW combined with the steep outwardly directed Na^+ gradient results in Na^+ loss which ultimately is the cause of death (Wilson and Taylor, 1993b).

Similarly, in marine fish Cu appears to disrupt osmoregulation during acute exposure. Marine fish must drink to replace fluid lost by diffusion to the concentrated environment and intestinal water absorption is driven by active absorption of Na^+ and Cl^-. The salt gained by this process and by diffusion across other body surface areas is extruded by active transport across the gills (Marshall and Grosell, 2005). Disruption of intestinal salt and water absorption, gill salt extrusion or both by Cu exposure appears to explain a Na^+ gain in SW. The effects of Cu on plasma Na^+ in the SW gulf toadfish display concentration dependence and are likely the cause of death during acute exposure (Grosell et al., 2004a,b).

Thus, it appears that at both salinity extremes, where Cu is most toxic to killifish, Cu disrupts the ability to sustain Na^+ gradients between the blood and the water which are steepest at these salinities. However, at intermediate salinities where toxicity is lower, there is little need for net Na^+ transport since Na^+ gradients are small or even absent (at ~12 ppt). Following this argument however, it may seem counterintuitive that fish are more sensitive in FW where the absolute Na^+ gradient is approximately 150 mM than in SW where the gradient is on the order of 300 mM. However, what may seem counterintuitive at first becomes reasonable when it is realized that the energy required to sustain ionic gradients relates to the relative rather than the absolute gradient. This fact is best illustrated by the Nernst equation which estimates, from electrochemical gradients, the energy required to sustain a given ion gradient across an epithelium or a membrane (Sten-Knudsen, 2002) (the equilibrium potential, E_p):

$$E_p = \left(\frac{RT}{zF}\right) 2.303 \log\left(\frac{Na_i^+}{Na_o^+}\right)$$

where $[Na_i^+]$ and $[Na_o^+]$ are the concentrations of Na^+ in the blood and water, respectively, z the valence of the ion in question and R, T, and F are the gas constant, absolute temperature and Faraday's constant, respectively. Since R, T, z and F are all constants, the energy required to sustain a chemical gradient relates to the concentration difference expressed as a ratio rather than as an absolute difference. Fig. 2B illustrates the LC50 values obtained from the killifish bioassays as a function of magnitude (not directionality) of the relative Na^+ gradient using actual measured Na^+ concentrations in the test solutions and an assumed extracellular fluid Na^+ concentration of 150 mM. The linear regression (Fig. 2B) indicates that 93% of the 50-fold variance in sensitivity to Cu can be accounted for by differences in Na^+ gradients. The same regression analysis was performed against ionic Cu^{2+} (from Table 2) to reveal an r^2 of 0.721 which is in sharp contrast to the 0.93 obtained from the regression analysis against total dissolved Cu. The bioassay data used for the regression analysis is strongly influenced by the freshwater data point and regression analysis was therefore performed also without the freshwater value. The result of this analysis was a similar slope (−0.009 compared to 0.013 for the full dataset and a r^2 of 0.39). The conclusion emerging from this data set therefore is that physiology rather than Cu speciation and competition are determining the relative sensitivity to Cu across salinities in this osmoregulatingfish.

4.2. Differences among species in Cu sensitivity in seawater

Variation in Cu toxicity among SW organisms is illustrated in Fig. 1. Observations may to some extent group phylogenetically with fish primarily occupying the more tolerant 50% and mollusks occupying predominantly the more sensitive 50%. Crustaceans appear to span the entire range while the few echinoderms tested all appear to be very sensitive. In addition to an apparent phylogenetic grouping, Fig. 1 also suggests a developmental grouping with the majority of adult test organisms displaying high tolerance and early life stages being more sensitive. Tests on juvenile organisms display an intermediate tolerance to acute Cu exposure.

The grouping according to life stages combined with the realization that size matters for acute Cu toxicity in FW organisms (Grosell et al., 2002) prompted an examination of the data for grouping according to size of the organism. Only a truncated data set was used for this analysis since many studies fail to provide information on mass, other size indices, life stage or age. For many of the studies we were able to include in this analysis we have estimated mass from length, condition factors or life stages (see figure legend for details) which is associated with uncertainty. Despite this uncertainty, considering all observations for which size could be estimated a significant correlation ($r^2 = 0.48$, $P < 0.01$) was observed with larger animals being the least sensitive. Four studies appear to show relatively high sensitivity despite relatively high mass of test organisms and include the adult long-spined sea urchin, *Diadema antillarum* (Bielmyer et al., 2005), the red abalone (*Haliotsis rufuscens* (USEPA, 2003), the black abalone (*Haliotsis cracherodii* (USEPA, 2003) and the bay scallop (*Argopecten irradians* (USEPA, 2003). These four studies suggest that there may be a phylogenetic signal in the variation in Cu sensitivity among species but that it is restricted to adults. What appears as a phylogenetic signal can be explained by the fact that most fish tested are relatively large while the majority of the sensitive mollusks, crustaceans and echinoderms are small (Fig. 3).

Nevertheless, while size explains much of the variation among studies, certain adult mollusks and echinoderms appear to be hypersensitive to acute Cu exposure relative to expectations based on size alone. However, in contrast to FW organisms which all osmoregulate and show size-dependent Na^+ turnover rates, the reasons for size-dependent Cu sensitivity in marine organisms may differ among species. While Na^+ turnover scales with size and the surface area:volume ratio in osmoregulating organisms (Grosell et al., 2002) this may not be the case for osmo- and iono-conformers which do not sustain a Na^+ gradient with respect to the environment. However, metabolic rate scales with size (Demetrius, 2006) which means that requirements for gas exchange, acid–base balance regulation and elimination of nitrogenous waste (ammonia in most aquatic organisms) also scale with size. Therefore, size-dependence of sensitivity in marine organisms could be related to not only Na^+ turnover rates, but parameters like gas exchange, acid–base balance and ammonia excretion. Variation in these physiological parameters, which are all sensitive to Cu, may account for differences in sensitivity in osmo- and iono-conforming organisms.

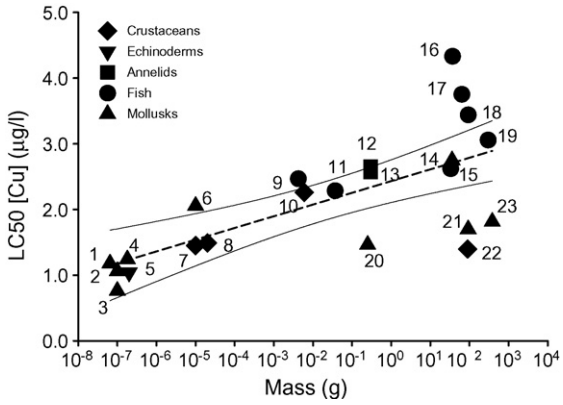

Fig. 3. Sensitivity to copper as a function of the mass of the organism. Diamonds represent crustaceans, downward triangles represent echinoderms, squares represent annelids, circles represent fish and upward triangles represent mollusks. Lines represent linear regression and confidence intervals ($r^2 = 0.481$, $P < 0.01$). In most cases, mass was estimated based on age, length, or lifestage. Unless stated otherwise, LC50 values used are taken from Table 1b of USEPA (2003). Larval masses estimated by assuming a sphere of the size listed below and a density of 1 g/cm^3. Individual points are as follows: #1, Eastern oyster (*Crassostrea virginica*) embryo. Mass estimated from egg size of 50 μm (Swann et al., 2006); #2, Pacific oyster (*Crassostrea gigas*) embryo. Mass estimated from egg size of 55–60 μm (McCombie et al., 2005); #3, Mussel (*Mytilus* spp.) embryo. Mass estimated from egg size of 60–65 μm (Behrends, 2002); #4, Blue mussel (*Mytilus edulis*) embryo. Mass estimated from egg size of 70 mm http://govdocs.aquake.org/cgi/reprint/2004/802/8020500.pdf); #5, *Diadema antillarum* larvae. Mass and LC50 data from Bielmyer et al. (2005); #6, Red abalone (*Haliosis rufescens*) larvae. Mass estimated from larval size of 250 μm (personal communication with Dr. Donal T. Manahan, USC); #7, *Artemia franciscana* cysts. Mass estimated from hydrated cyst diameter of 250 μm (http://www.artemia-international.com/); #8, *Acartia tonsa* (Miller et al., 1977); #9, *Fundulus heteroclitus*, present study (4.2 mg). Mass used is the mean weight of 10 7-day-old juveniles; #10, Mysid (*Americamysis bahia*) age 24 h. Mass estimated based on dimensions of 24 h mysid (Wortham-Neal and Price, 2002); #11, *F. heteroclitus* juveniles (Grosell, Cai, Solo-Gabrielle, Gerdes, unpublished); #12 and #13, *Nereis diversicolor* adult. LC50 from Jones et al. (1976) and mass estimated at 0.3 g; #14, Pacific oyster (*C. gigas*) adult. Mass estimate from (Kobayashi et al., 1997); #15, Shiner perch (*Cymatogaster aggregate*) adult. Mass estimated from length (Harvey et al., 2000); #16, Toadfish (*Opsanus beta*) adult. LC50 and mass from Grosell et al. (2004b); #17, Atlantic croaker (*Micropogon undulates*). Mass estimated from length (Wigley et al., 2003); #18, Pinfish (*Langodon rhomboids*). Mass calculation from http://fwie.fw.vt.edu/WWW/macsis/lists/TSNl0106.htm; #19, Sheepshead (*Archosargus probatocephalus*). Mass calculated from length (Schwartz, 1990); #20, Bay scallop (*Argopecten irradians*). Mass calculated from length (Kellogg et al., 1988); #21, Black abalone (*Haliotis cracherodii*). Mass calculated from size (Mcshane et al., 1988); #22, *D. antillarum* adult. LC50 and mass from Bielmyer et al. (2005); #23, Red abalone (*Haliotis rufescens*). Mass calculated from size (Mcshane et al., 1988).

Finally, some of the most sensitive tests are based on developmental endpoints (USEPA, 2003) which may suggest that Cu acts as a developmental toxicant. While this may be true, the possibility exists that impaired respiration, or other specific physiological endpoints may result in slowed or even arrested development as a secondary effect.

4.3. Conformers versus regulators—mechanisms of toxicity?

While Na^+ gradients are powerful in explaining differences in sensitivity to Cu within a single fish species, no

apparent difference in sensitivity to acute exposure is seen between osmoregulating and osmoconforming animals. Under the assumption that Cu is an osmoregulatory toxicant this is surprising since osmoconformers exhibit no or limited Na^+ gradients with respect to the environment. In contrast, osmoregulating fish maintain relatively high Na^+ gradients and rely on Na^+ transport. From these considerations, an expectation of osmoconforming organisms in SW to be more tolerant than osmoregulating fish seems reasonable. However, the available data lends no support to this expectation since organisms fall on the same regression line regardless of osmoregulatory strategy, which prompted us to question the assumption that Cu is an osmoregulatory toxicant in saline environments.

4.4. Mechanisms of Cu toxicity in marine fish and invertebrates

Only a few detailed studies have focused on the physiology of fish exposed to acutely lethal or near lethal Cu concentrations and these reveal osmoregulatory disturbances (Grosell et al., 2004a,b). The gulf toadfish showed evidence of impaired intestinal and possibly branchial ion transport physiology and demonstrated Cu induced drinking rate disturbances (Grosell et al., 2004a,b). While these studies agree with work performed on flounder and trout (Stagg and Shuttleworth, 1982; Wilson and Taylor, 1993a) other studies have reported acid–base balance and ammonia excretion disturbances without evidence of osmoregulatory impairment, at least in one case leading to Cu induced mortality (Blanchard and Grosell, 2006; Larsen et al., 1997). On balance, it seems that osmoregulatory failure in most cases is the cause of Cu induced mortality.

It is puzzling that marine invertebrates which often are osmoconformers in SW are as sensitive as osmoregulating fish since no net ion gradients need to be maintained by these animals. For osmoconforming marine invertebrates, maintenance of net Na^+ gradients cannot be the target for acute Cu toxicity; other physiological processes must be influenced during exposure. Surprisingly few studies have addressed the mechanisms of toxicity in marine invertebrates but thorough and classic studies of responses to Cu exposure in the shore crab, *Carcinus maenas* provide valuable information (Boitel and Truchot, 1988, 1989, 1990). In the shore crab in SW, acute Cu exposure results in systemic acid–base balance disturbance presumably caused by restriction of gas exchange capacity without causing changes in composition of the haemolymph. A similar response was recently reported for the long-spined sea urchin, *D. antillarum*, which displayed a compensated acidosis and slight osmoregulatory disturbance at low concentrations but a pronounced acidosis and lack of osmoregulatory disturbance at lethal concentrations (Bielmyer et al., 2005). Impaired gas exchange induced by Cu exposure may explain these observations. At the high concentrations employed in the study on crabs, the cause of respiratory impairment might have been increased diffusive distance due to general epithelial damage. However, in the sea urchin study low concentrations were employed (25 μg/l) and may relate to inhibition of the enzyme carbonic anhydrase. Carbonic anhydrase facilitates rapid equilibration between molecular CO_2 and HCO_3^- and serves gas exchange and acid–base balance regulation (Henry et al., 1995; Henry et al., 1997). Indeed, carbonic anhydrase from the estuarine crab, *Chasmagnathus granulate* is sensitive to Cu (Skaggs and Henry, 2002; Vitale et al., 1999) indicating that this enzyme may be central to Cu induced acid–base balance disturbance. Interestingly, acid–base balance disturbance, likely related to respiratory impairment has been reported for marine fish (Larsen et al., 1997; Wilson and Taylor, 1993a) suggesting a commonality among marine invertebrates and fish.

The most consistent response to Cu exposure in fish is the impairment of ammonia excretion (Blanchard and Grosell, 2006; Grosell et al., 2003, 2004b; Wilson and Taylor, 1993a). However, the exact mechanisms for ammonia excretion remain to be elucidated and it is unclear (but cannot be excluded) whether impaired ammonia excretion is responsible for Cu induced mortality.

Having now considered osmoregulating fish and osmo- and iono-conforming invertebrates, one group of osmoconforming but iono-regulating animals, the elasmobranchs (sharks, skates and rays) remains to be discussed. Since these animals regulate blood salt content below that of SW, they actively maintain a Na^+ gradient. However, plasma NaCl levels are much higher than in teleost fish (280 mM compared to 150 mM, respectively). Under the assumption that lethality from Cu exposure occurs due to osmoregulatory disturbance, elasmobranchs would thus be predicted to be less sensitive than teleost because their relative Na^+ gradient is less. However elasmobranchs are generally perceived as being sensitive to metal exposure which correlates well with an apparent hyper-accumulation of metal compared to teleost fish (De Boeck et al., 2001; Grosell et al., 2003; Pentreath, 1977; Webb and Wood, 2000). A single study to date have evaluated the mode of toxic action of Cu in elasmobanchs (De Boeck et al., 2007) and an earlier study revealed reduced ammonia excretion during acute exposure without evidence of osmoregulatory disturbance in the clearnosed skate, *Raja erinacea* (Grosell et al., 2003). This latter study of sublethal effects thus confirms observations from teleosts that reduced ammonia excretion may be a more sensitive endpoint (Grosell et al., 2003) while the most recent study demonstrate acid–base balance disturbance, respiratory impairment as well as osmoregulatory disturbance at higher Cu concentrations (De Boeck et al., 2007).

In marine iono-conformers where iono-regulatory effects cannot be the mode of action, the principal cause of mortality as a result of acute exposure appears to be either acid–base balance disturbance related to impaired respiratory gas exchange and/or ammonia excretion. These effects are also observed in marine fish (both teleosts and elasmobranchs) and often are more sensitive endpoints than osmoregulatory disturbances (Grosell et al., 2003, 2004b). A common denominator for acid–base balance and ammonia excretion is the enzyme carbonic anhydrase which has been reported to be inhibited by Cu exposure in crustaceans (Skaggs and Henry, 2002; Vitale et al., 1999) but so far not in fish (Blanchard and Grosell, 2006).

4.5. Intermediate salinities—physiological diversity

Moving from SW to lower, intermediate salinities likely comprise an even more complicated situation with respect to the identification of unifying principles predicting sensitivity to Cu. While we have presented evidence that ionic gradients for teleost fish account for much of the salinity induced difference in sensitivity to Cu, the multiple osmoregulatory strategies employed by marine and estuarine organisms confound matters. Some invertebrates and most elasmobranchs maintain internal osmotic pressure similar to that of full strength SW regardless of ambient salinity and thus assume an iono-regulatory strategy at lower salinities.

Most invertebrates that occur only in fully marine environments are osmo- and iono-conformers and are iso-osmotic at 1000–1100 mOsm (Robertson, 1949). When exposed to more dilute salinities, they continue to osmo- and iono-conform, normally reaching a lethal limit in the range of 500–700 mOsm (∼15–21 ppt salinity) (Charmantier et al., 2001). Estuarine invertebrates are extremely variable in their osmo- and iono-regulatory capabilities. They are typically iso-osmotic at some point between 300 and 900 mOsm. Above their iso-osmotic point, most estuarine invertebrates are osmo- and iono-conformers although a few (particularly decapod crustaceans) are capable of hypo-regulation (Spaargaren, 1972). Typically species that have relatively high iso-osmotic points (e.g., 600–900 mOsm), weakly hyper-regulate below their iso-osmotic point until their extracellular osmolality reaches ∼300–350 mOsm, at which point they begin to strongly osmo- and iono-regulate (Henry, 2001; Smith, 1970).

A few estuarine invertebrates are capable of osmo- and iono-conforming over their full tolerance range. An extreme example of this is the euryhaline polychaete *Mercierella enigmatica* which osmo- and iono-conforms over a remarkable range of 43–1640 mOsm in the external environment (Skaer, 1974a,b)

4.6. Intermediate salinities—implications for Cu sensitivity

Assuming that osmoregulatory disturbance is the cause of mortality during acute Cu exposure in animals which maintain an ionic gradient and that impaired acid–base balance and/or reduced ammonia excretion leads to fatality in absence of ionic gradients, the mechanism of Cu toxicity to single species will depend on salinity. Furthermore, the salinity point at which a shift from one mode of action to another occurs will depend on the osmotic set point of the organism. For teleost fish, isoosmotic conditions occur ∼12 ppt but for elasmobranchs, no Na^+ gradient would exist close to 18 ppt. For invertebrates maintaining internal osmotic pressure at 1000 mOsm, the shift from acid–base balance/ammonia excretion to osmoregulatory effects would occur at very high salinities.

The suggested shift in targets for acute Cu toxicity is illustrated by a study performed on the shore crab, *C. maenas*, which is an osmoconformer in SW but an osmoregulator at lower salinities (Boitel and Truchot, 1990). An acute Cu effect on shore crabs in SW and 50% SW (∼17 ppt) was acid–base balance disturbance. In contrast, osmoregulatory disturbance only occurred at 17 ppt where the crabs actively regulate Na^+ gradients. Interestingly, mortality in response to Cu only occurred at 17 ppt where osmoregulation was targeted. While this may suggest that osmoregulatory failure is the principal cause of death, it should be noticed that the degree of the acid–base balance disturbance was greater at 17 ppt than in SW (Boitel and Truchot, 1990). Regardless of the cause of mortality, sensitivity to Cu in the shore crab correlates well with the Na^+ gradient maintained between the extracellular fluid and the environment as was the case for killifish in the present study.

A review of the literature revealed eight studies on the influence of salinity on acute Cu toxicity in fish and invertebrates which are summarized in Fig. 4. Although none of the studies span the entire range of salinities, all studies have one thing in common: that the highest sensitivity occurs at the lowest salinity. However, it is important to realize that none of the studies show a linear increase in tolerance with increasing salinity as competitive interactions among cations would dictate and that none of the studies reflects the Cu speciation calculated in the present study (Table 2). While all studies show the highest sensitivity at the lowest tested salinity it is also clear that the salinity threshold for increased sensitivity differs greatly among species. While the three tests on *N. diversicolor* (now *Hediste diversicolor*) uniformly demonstrate highest tolerance at ∼17 ppt, the American lobster shows highest tolerance at 25 ppt, while the striped bass shows highest tolerance at 10 ppt (which is in agreement with the present study). The two mollusks demonstrate diversity with the

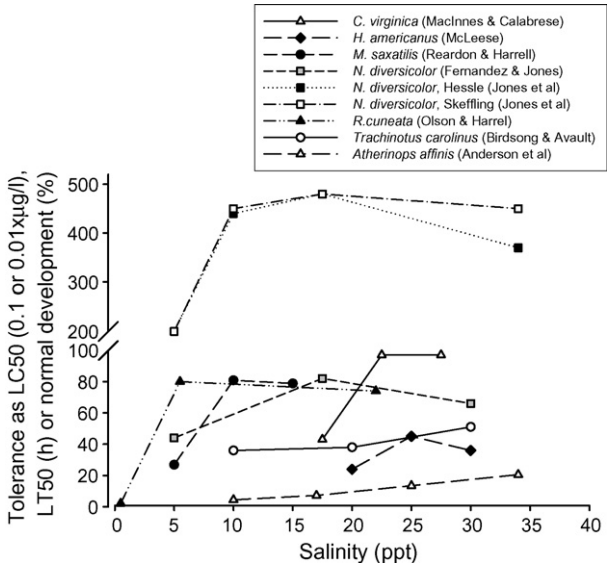

Fig. 4. Summary of the available literature reporting tolerance to copper at different salinities. Units are either LT50 (h), LC50 (0.1 or 0.01 μg/l) or % normal development. Triangles pointing upwards represent mollusks, triangles pointing downwards represents echinoderms, diamonds represent crustaceans, circles represent fish, while squares represents annelids. Data presented are from the American oyster (*Crassostrea virginica*) at 25 °C (Macinnes and Calabrese, 1979), the American lobster (*Homarus americanus*) exposed to 560 μg/l at 13 °C (Mcleese, 1974), striped bass (*Morone saxatilis*) (Reardon and Harrell, 1990), the polychaete, *Neries diversicolor* (Fernandez and Jones, 1990; Jones et al., 1976), the clam, *Rangia cuneata* (Olson and Harrel, 1973), the pompano, *Trachinotus carolinus* (Birdsong and Avault, 1971) and the larval topsmelt, *Atherinops affinis* (Anderson et al., 1995).

oyster showing highest tolerance above 22 ppt and the brackish water clam demonstrating peak tolerance at 5 ppt (Fig. 4). With the hypersensitivity of certain adult mollusks and echinoderms in SW (Fig. 3) studies of the influence of salinity on Cu toxicity in these or related species seems to be called for.

It appears that a general prediction regarding salinity and acute Cu toxicity is that lower salinity or greater internal-to-external Na^+ gradients results in highest sensitivity. It is important to keep in mind that for fish, large Na^+ gradients are maintained at both low- and high-salinities while Na^+ gradients in invertebrates, when maintained generally increase with decreasing salinity. It is also important to realize that the threshold at which many invertebrates switch from osmoconformity (in SW) to osmoregulation differs among species. In the salinity range in which a given organism osmoconforms, salinity will likely only influence sensitivity through Cu speciation and competition but sensitivity will probably always increase with decreasing salinity, in salinities where the same organisms osmoregulate.

5. Conclusions

In conclusion, the magnitude of Na^+ gradients appears to be the key parameter influencing relative sensitivity to Cu in osmoregulating organisms. Furthermore, difference in size accounts for a substantial fraction of the variation among species although certain adult mollusks and echinoderms seems hypersensitive relative to their size. Finally, we conclude that the diversity of osmoregulatory physiology among organisms inhabiting saline environments makes for an exciting challenge for scientists interested in establishing unifying principles of interactions between Cu toxicity and environmental salinity—much work remains to be done.

6. Recommendations for further/future research

Size influences relative sensitivity to Cu both in FW and SW and it is therefore important that an indication of size of test organisms is included with any toxicity or physiology study. Furthermore, detailed water chemistry and relevant physiological parameters should be reported whenever possible.

The cause of mortality in osmoconforming organisms is unknown and investigations of the physiological mode of action responsible for Cu toxicity in the diverse and important group of invertebrates and elasmobranchs clearly are needed. Such investigations ought to be comparative in nature to also address the uncertainty associated with the hypersensitivity seen in certain adult mollusks and echinoderms (Fig. 3).

The most consistent response to Cu exposure regardless of salinity is impaired ammonia excretion. Yet, the mode of ammonia excretion in marine environment is at best poorly understood and nothing is known about how Cu interacts with this important metabolic process. This challenging uncertainty deserves attention.

Although truly euryhaline echinoderms are rare, many tidal zone species experience fluctuations in salinities as do many mollusks. Considering the hypersensitivity to Cu (Fig. 3) of these invertebrates in SW it seems critical that the influence of salinity on Cu tolerance in these animals is investigated.

The present paper has evaluated and discussed the influence of salinity on Cu sensitivity but has strictly focused on constant salinities. Coastal zones are characterized by fluctuating salinities and to our knowledge no studies have examined the influence of salinity fluctuations on Cu sensitivity. Conversely, no studies have examined the influence of Cu on the ability of organisms to tolerate the rapid salinity changes typical of tidal environments. The need to address these unknowns seems eminent.

Along similar lines of thought, certain tidal zone animals are exposed to air during low tide with resulting demands for physiological compensation. The physiological processes involved with this lifestyle, which includes ammonia accumulation and subsequent fast excretion upon re-immersion (Durand and Regnault, 1998; Regnault, 1992), likely are sensitive to Cu, yet no studies have examined this possibility.

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