Accepted Manuscript

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PII: S0141-1136(08)00160-8
DOI: [10.1016/j.marenvres.2008.05.006](https://doi.org/10.1016/j.marenvres.2008.05.006)
Reference: MERE 3258

To appear in: *Marine Environmental Research*

Received Date: 27 March 2007
Revised Date: 4 January 2008
Accepted Date: 14 May 2008

Please cite this article as: Rosen, G., Rivera-Duarte, I., Chadwick, D.B., Ryan, A., Santore, R.C., Paquin, P.R., Critical tissue copper residues for marine bivalve (*Mytilus galloprovincialis*) and echinoderm (*Strongylocentrotus purpuratus*) embryonic development: conceptual, regulatory and environmental implications, *Marine Environmental Research* (2008), doi: [10.1016/j.marenvres.2008.05.006](https://doi.org/10.1016/j.marenvres.2008.05.006)

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**Critical tissue copper residues for marine bivalve (*Mytilus galloprovincialis*) and
echinoderm (*Strongylocentrotus purpuratus*) embryonic development: conceptual,
regulatory and environmental implications**

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ABSTRACT

Critical tissue copper (Cu) residues associated with adverse effects on embryo-larval development were determined for the Mediterranean mussel (*Mytilus galloprovincialis*) and purple sea urchin (*Strongylocentrotus purpuratus*) following laboratory exposure to Cu-spiked seawater collected from San Diego Bay, California, USA. Whole body no-observed-effect-residues (NOER) were similar, with means of 21 and 23 $\mu\text{g g}^{-1}$ dw, for *M. galloprovincialis* and *S. purpuratus*, respectively. Mean whole body median effect residues (ER50) were 49 and 142 $\mu\text{g g}^{-1}$ dw for *M. galloprovincialis* and *S. purpuratus*, respectively. The difference in ER50s between species was reduced to a factor of <2 when expressed as soft tissue residues. Coefficients of variation among whole body-ER50s were 3-fold lower than median waterborne effect concentrations (EC50) for both species exposed to samples varying in water quality characteristics. This suggests that tissue concentrations were a better predictor of toxicity than water concentrations. The CBRs described herein do not differentiate between the internal Cu concentrations that are metabolically available and those that are accumulated and then detoxified. They do appear, however, to be well enough related to the level of accumulation at the site of action of toxicity that they serve as useful surrogates for the copper concentration that affects embryonic development of the species tested. Results presented have potentially important implications for a variety of monitoring and assessment strategies. These include regulatory approaches for deriving saltwater ambient water quality criteria for Cu, contributions towards the development of a saltwater Biotic Ligand Model, the conceptual approach of using CBRs, and ecological risk assessment.

Keywords: copper, body residue, Biotic Ligand Model, embryo, larval bioassay, *Mytilus galloprovincialis*, *Strongylocentrotus purpuratus*, water quality

1. Introduction

Copper (Cu) is frequently measured at elevated concentrations in coastal environments. Its dissolved bioavailability and toxicity to aquatic organisms, however, is dependent upon its physico-chemical speciation (Morel, 1983; Campbell, 1995; Allen and Hansen, 1996), which is determined largely by the presence of different organic and inorganic ligands in a given water body (Allen and Hansen, 1996), as well as biological factors such as size, life stage and whether or not dietary uptake is a potential pathway for exposure. Although there is a preponderance of evidence that free aqueous Cu ion is the most biologically available, and therefore potentially toxic, Cu species available for dissolved uptake (Sunda and Guillard, 1976; Zamuda and Sunda, 1982), ambient water quality criteria (WQC) are currently expressed as dissolved metal (USEPA, 1995a). In addition, WQC are typically developed from toxicity tests conducted in laboratory water that is generally low in metal binding ligands such as dissolved organic carbon (DOC; USEPA, 1985). Therefore, national criteria may be either over-protective or under-protective, depending on site-specific characteristics. Water effect ratio (WER) studies, which involve concurrent toxicity tests with site and laboratory waters, provide a ratio that can be multiplied by the national criterion in order to make it site-specific (USEPA, 1994; USEPA, 2001). WER studies conducted on coastal water bodies have resulted in increases of national WQC for Cu by factors ranging from ~1.5 (e.g. USEPA, 1994; CH2MHill, 2000; Rosen et al., 2005) to 2.7 (City of San Jose, 1998). Although WER studies have proven useful for setting site-

specific WQC, they involve labor intensive and costly toxicity testing and associated chemical analyses in order to characterize spatial and temporal differences in the relative dissolved bioavailability and toxicity of Cu at the site.

In freshwater settings, results of WER studies have been successfully predicted using the Biotic Ligand Model (BLM). This approach calculates the critical accumulation of dissolved metal at the site of toxic action using commonly measured water quality parameters such as temperature, dissolved organic carbon (DOC), pH, alkalinity, salinity, Ca, Mg, Na, K, SO_4^{2-} , and Cl^- (USEPA, 2007). This approach is also more cost-effective than toxicity testing (Santore et al., 2001; Santore, et al., 2003). The BLM is based on the prediction of metal accumulation by a biotic ligand (e.g. fish gill) that accumulates metal ions from the exposure water. The amount of bound metal is determined by a competition for metal ions with other aqueous ligands, particularly dissolved organic matter (DOM), and the competition for the biotic ligand between the bioavailable forms of the stressor metal and other cations in solution. The utility of the BLM approach to derive site-specific acute water quality criteria for Cu is demonstrated by its incorporation into an updated freshwater WQC document (USEPA, 2007).

For many aquatic invertebrates, the site of toxic action is unknown, or is not accessible for direct measurement. Critical body residues (CBR), based on whole body concentrations, have been suggested as a more toxicologically relevant means of predicting toxicity as compared to water concentrations for a variety of non-polar organic contaminants and metals (McCarty and Mackay, 1993). One of the difficulties in applying the CBR

approach to metals, however, involves the potential for differences among organisms to compartmentalize, detoxify, and eliminate the nonessential fraction of accumulated metal (McGeer et al., 2003; Vijver et al., 2004; Luoma and Rainbow, 2005). Although the actual metabolically available concentration of a metal causing toxicity may be the same or similar among species, whole body CBRs may not accurately reflect toxicity due to the species-specific differences in the ability to detoxify and store accumulated metal.

The purpose of this study was to determine if tissue Cu concentrations could be related to toxic effects in early life stages of select marine invertebrates exposed in the laboratory. Embryo-larval development testing of marine invertebrates often plays a critical role in the development of WQC for Cu in saltwater. Identification of a critical accumulation level, could be used to provide calibration data for development of a BLM for seawater, as well as provide a possible alternative approach for monitoring and assessment studies. The endpoints investigated were embryo-larval development of the Mediterranean mussel (*Mytilus galloprovincialis*) and purple sea urchin (*Strongylocentrotus purpuratus*). These endpoints were selected because of their role as drivers of the current saltwater WQC for Cu ($3.1 \mu\text{g dissolved Cu L}^{-1}$; USEPA, 1995a) due to effects at very low concentrations (USEPA 1995a, 2003). Because the life stages used are considered non-feeding (Sprung, 1984; Fenaux et al., 1985), concerns about the sometimes overlooked role of dietary uptake in metal accumulation (Luoma, 1995; Fisher and Hook, 2002) were reduced. Cu was spiked into seawater samples collected from different locations and seasons in San Diego Bay, CA, USA to demonstrate the effects of varying water quality characteristics on Cu accumulation and toxicity.

2. Methods

2.1 Sample collection and treatment

Natural surficial (~ 1 m depth) seawater was collected from the mouth of San Diego Bay (North) and from the southern interior portion of the bay (South; Figure 1). These two sites are non-toxic to bivalve and echinoderm embryos, yet possess different water quality characteristics (e.g. DOC and dissolved Cu concentration) that have been previously demonstrated to correlate with differing degrees of Cu bioavailability (Blake et al., 2004; Rivera-Duarte, et al., 2005; Rosen et al., 2005). Seawater was sampled, and filtered to 0.45 µm in the field, using clean techniques (USEPA, 1995b). Samples were held at 4 °C after collection and used within 24 h of collection.

2.2 Embryogenesis Exposures

Exposures were conducted according to a slightly modified version of standard toxicity testing procedures for evaluating embryo-larval development success with echinoderms and bivalves (USEPA, 1995c). Modifications to the standard procedures were as follows. In order to obtain enough biomass for weight measurements and tissue analyses, experiments were conducted at higher initial embryo concentrations (60 and 40 embryos mL⁻¹, for mussels and sea urchins, respectively). Additionally, larger water volumes (750 mL in 1L polypropylene beakers) were also used. These test conditions were evaluated in preliminary experiments that showed no adverse effects on controls or changes to Cu sensitivity. Typically, seven concentrations of Cu bracketing the expected EC50, were prepared by adding Cu (as CuSO₄•5H₂O) to the San Diego Bay water samples. A period

of 2 to 3 hours was allowed for equilibration of the added Cu prior to the addition of embryos. A filter blank was also included that consisted of unspiked seawater and no embryos. All concentrations were tested in triplicate.

Adult mussels were obtained from Carlsbad Aquafarm (Carlsbad, California, USA), where the mussels were cultured under continuously submerged conditions in an embayment about 20 miles north of San Diego. Sea urchins were field collected subtidally from local field populations by Marinus Scientific (Garden Grove, California, USA). Mussels were induced to spawn in the laboratory by thermal shock, which involved raising the water temperature by ~10 °C, while sea urchin spawning was induced by injection of 0.5 M KCl into the peristomal membrane (USEPA, 1995c). Within 4 h of fertilization, embryos were added to test solutions, which were covered, and held on a 16:8 h light:dark cycle at 15 °C in temperature controlled light chambers (Percival Scientific, Model 136LL). Because the embryos are lecithotrophic (yolk supply provides nourishment), test animals were not fed during the exposures, thereby eliminating the dietary exposure pathway. Mussel and sea urchin embryos were allowed to develop for 48 and 96 hours, respectively, prior to termination of the experiments.

2.3 Water quality measurements

Water quality parameters including pH, temperature, dissolved oxygen, and salinity were measured daily from surrogate beakers that also included embryos. Dissolved organic carbon (DOC) was determined on unspiked seawater samples according to U.S. EPA Method 415.1.

2.4 Larval toxicity assessment

After the appropriate exposure time, each beaker was gently homogenized by swirling with a disposable plastic pipette, and a 5 ml aliquot removed and preserved in 500 μ l of concentrated formaldehyde in glass scintillation vials for microscopic evaluation of larval development success. The percentage of normal larvae was determined by counting the first 100 larvae encountered using an inverted microscope at a magnification of 40x. After the designated exposure period, normal mussel larvae have achieved the prodissococonch I stage, characterized by a straight-hinged D-shaped larval shell. Normal sea urchin larvae have reached the pluteus stage, which is pyramidal in shape with four well developed skeletal rods (Figure 2). Because the final larval weight was required for the tissue calculations, final larval weight was also used as a toxicity endpoint.

2.5 Whole body residue determination

After removing the toxicity aliquot, the remainder of each sample (~745 mL) was filtered through an acid-cleaned and pre-weighed 8 μ m polycarbonate filter in a class-100 clean room. The filtrate was set aside for dissolved Cu measurements (see Section 2.7), which was used to derive water-based toxicity metrics. The filter (containing the larvae) was rinsed with ~100 mL of 18M Ω /cm water to remove loosely bound Cu (Playle et al., 1992), and then placed into an acid-cleaned 2 mL polypropylene centrifuge tube. For tissue try weight determination, the contents of the tube were dried at ~25 °C on a hot plate set inside a class 100 all-polypropylene fume hood, until a constant weight was achieved (~48 h). After recording the mass, the sample was digested with 100 μ L Quartz-still grade HNO₃

(Q-HNO₃) overnight. The next day, 1 mL 18MΩ/cm water was added, the tube capped, and the sample set aside for Cu measurements (see Section 2.7).

2.6 Soft tissue residue determination

Soft tissue Cu concentrations were estimated in a side experiment conducted concurrently with Experiment #3 using North Bay water samples. Whole body residues were measured as previously described (Section 2.5) for 3 replicates, and a duplicate set of 3 replicates was treated with NaOH to digest the tissues, but leave the calcareous parts (larval shell or skeletal rods) intact. After the initial dry weight determination, 250 µL 1 N NaOH was added to the tube. The tube was capped and contents digested overnight. The contents of the tube (including the filter), were then transferred to a new pre-weighed filter, using ~200 mL 18MΩ/cm water for transfer and rinsing. The new filter was allowed to dry for 48 h on a hot plate (~25 °C) for dry weight determination. The sample was then returned to a clean centrifuge tube and acidified with 100 µL Q-HNO₃ and allowed to digest overnight. The next day, 1 mL 18MΩ/cm water was added to result in a final concentration of 1 N Q-HNO₃, which was stored for Cu analysis (see Section 2.7). The soft tissue concentration was calculated as described in Section 2.8.

2.7 Cu Analysis

Dissolved ambient Cu concentrations were measured following clean techniques. Cu in the samples was preconcentrated following the liquid:liquid carbamates preconcentration procedure of Bruland et al. (1985). A recovery of 96% (0.57 µg L⁻¹ out of 0.592 ± 0.055 µg L⁻¹) in the preconcentration was measured for the standard reference material (SRM)

CASS4 (coastal seawater) from the National Research Council of Canada. Cu concentrations in the filtrate and digested tissues were measured by STGFAA spectroscopy by appropriate dilution in 1N Q-HNO₃ and direct injection. A 100% accuracy with a precision of 10% was measured for SRM 1643d (trace metals in water) of the National Bureau of Standards in the STGFAA analysis.

2.8 Data analysis

Both exposure water- and residue-based toxicity metrics were calculated with ToxCalc software (Tidepool Scientific) using measured water and tissue concentrations. Water concentrations, therefore, incorporated both background and added Cu. The no-observable-effect-concentrations and residues (NOEC, NOER) were determined using hypothesis testing following confirmation of normal distribution of data and equal variances on arc-sine square root transformed data. Median effects concentrations and residues (EC50, ER50) were determined using Probit point estimation. Sigmoid, 3 parameter curves were used to fit dose responses with Sigma Plot (version 8.0).

Soft tissue concentrations were estimated using:

$$C_{st} = \frac{C_{wb} * M_{wb} - C_s * M_s}{M_{st}} \quad (2)$$

where, C_{st}= Cu concentration in soft tissue (µg g⁻¹ dw), M_{wb} = mass of whole body (µg dw), C_s = Cu concentration in shell (µg g⁻¹ dw), M_s = mass of shell (µg dw), and M_{st} = mass of soft tissue (µg dw).

3. Results

3.1 Water quality characteristics

A summary of water quality data for the unspiked (no Cu added) samples is provided in Table 1. Parameters varied minimally, with South samples having slightly higher average pH and salinity values compared to North samples. DOC concentrations averaged 1.82 ± 0.59 and 2.84 ± 0.84 mg L⁻¹ for North and South samples, respectively. Dissolved Cu concentrations in unspiked North and South samples, which served as the controls for the experiments, averaged 0.8 ± 0.2 and 2.5 ± 0.0 µg L⁻¹, respectively.

3.2 Water-based toxicity metrics

Cu additions resulted in toxicity at varying water concentrations (Figure 3). Toxicity metrics based on the exposure water concentrations are provided in Table 2 and Figure 4a. *M. galloprovincialis* EC50 values varied approximately 2-fold, with the lowest EC50s being observed for North Bay samples, and the highest for the South Bay sample. A similar relationship was observed for the *S. purpuratus* experiment, but EC50s varied somewhat less (factor of 1.4). The coefficient of variation (CV) among EC50s was 35.1 and 25.5% for *M. galloprovincialis* and *S. purpuratus*, respectively.

M. galloprovincialis EC50s were positively correlated with DOC concentration ($r^2 = 0.995$; $p = 0.048$, $n=3$). For experiments 2 and 3, in which North and South samples were evaluated concurrently, EC50s differed by factors of 1.47 and 1.44 for *M. galloprovincialis*

and *S. purpuratus*, respectively, while DOC concentrations between the two sample locations differed by similar factors of 1.32 and 1.38.

A strong correlation was observed between *M. galloprovincialis* EC50 values calculated from normal larval development data and EC50 values calculated using the final larval weight data ($r^2=0.952$). As a toxicity endpoint, *M. galloprovincialis* larval weight was slightly less sensitive than normal larval development, with EC50 values averaging $23.5 \pm 11.5\%$ higher than those calculated using normal larval development (Figure 5). Change in weight of the developing mussel embryos over time from a side experiment are shown in Figure 6. *S. purpuratus* larval weight EC50s could not be generated due to the low sensitivity of this endpoint.

North Bay Cu EC50 values for both *M. galloprovincialis* and *S. purpuratus* were within our laboratory's control chart limits for reference toxicity tests conducted using filtered, natural coastal seawater similar in characteristics to North Bay. This suggests that the organisms used in this study exhibited typical sensitivity from Cu exposure.

3.3 Tissue based toxicity metrics – Whole body residues

Whole body residues in controls (no Cu added) tested in North Bay seawater averaged $4.61 \pm 2.01 \mu\text{g g}^{-1} \text{ dw}$ for *M. galloprovincialis* and were $3.63 \mu\text{g g}^{-1} \text{ dw}$ for the one *S. purpuratus* sample (Table 2). Whole body concentrations in South Bay seawater controls were somewhat higher than North Bay for *M. galloprovincialis* but no different for *S. purpuratus* (Table 2).

As the water Cu concentration increased, whole body residues of both *M. galloprovincialis* and *S. purpuratus* increased (Figure 3), but differed proportionally among individual experiments and between sampling locations (Figure 7). For a given dissolved Cu concentration, higher tissue concentrations were inversely proportional to DOC concentration (Table 1).

Toxicity metrics based on whole body residue concentrations are shown in Table 2 and Figure 4b. *M. galloprovincialis* ER50 values were similar among the experiments, averaging $49.2 \pm 4.8 \mu\text{g g}^{-1} \text{ dw}$. The 95% confidence limits around the ER50s overlapped for all samples. *S. purpuratus* ER50 values were similar among the experiments, averaging $142 \pm 15.6 \mu\text{g g}^{-1} \text{ dw}$, with the 95% CL once again overlapping. The CV among ER50s was 9.7 and 11.0% for *M. galloprovincialis* and *S. purpuratus* experiments, respectively, indicating less variability among ER50s compared to EC50s. NOERs for *M. galloprovincialis* and *S. purpuratus* differed by a factor of 1.1, averaging 20.6 and 23.3 $\mu\text{g g}^{-1} \text{ dw}$, respectively (Table 2).

3.4 Soft tissue residues

Comparison of tissue dry weights from NaOH digested and non-NaOH digested samples from the same exposure concentrations indicated that the calcareous structures (Figure 2) of *M. galloprovincialis* (shell) and *S. purpuratus* (skeletal rods) made up approximately 46 and 21% of the final whole body weight in control treatments. The relative fraction of shell or skeletal rod weight decreased at toxic exposure concentrations, which was

expected in the mussels due to the lack of shell development at concentrations above the EC50.

Although the calcareous structures are a substantial portion of the whole body weight, Cu residues in these structures were almost negligible, averaging only 7.9 and 3.7% of the total residue measured in the whole body for *M. galloprovincialis* and *S. purpuratus*, respectively. This resulted in estimates of soft tissue Cu residues that were higher than whole body residues by a factor of 1.6 and 1.2 for *M. galloprovincialis* and *S. purpuratus*, respectively (Table 3). Compared to whole body ER50s, soft body ER50s differed less between the species, dropping from a factor of 2.5 to 1.8.

4. Discussion

4.1 Toxicity expressed using water concentrations

These findings substantiate previous work demonstrating both temporal and spatial variability in potential Cu toxicity in San Diego Bay surface water samples. More specifically, this has been demonstrated for embryos of both *M. galloprovincialis* and *S. purpuratus*, when expressed on the basis of dissolved water concentrations (Blake et al., 2004; Rivera-Duarte et al., 2005; Rosen et al, 2005). In the current experiments, we calculated EC50s that differed by a factor of 2.0 for mussel embryos and a factor of 1.4 for sea urchin embryos.

Final larval tissue weight served as an additional toxicity endpoint for *M. galloprovincialis*, proving to be nearly as sensitive as the more traditionally used normal development endpoint. This is not surprising, as inhibited formation of the relatively heavy larval shell (46% of total weight in controls at 48 h of development) at toxic concentrations implies that the larvae would not achieve the straight hinged D-shape indicative of normal development. Such a relationship was not apparent in the *S. purpuratus* experiments, likely because the skeletal rods of the pluteus are not as significant a factor in the overall mass. In addition, the skeletal structures could still be present, albeit deformed, in abnormal specimens.

The differences in EC50 values between North and South San Diego Bay have previously been attributed to differing water quality characteristics such as total suspended solids (TSS) and DOC concentrations, which tend to be higher in South Bay (Blake et al., 2004; Rivera-Duarte et al., 2005; Rosen et al., 2005). These parameters affect Cu speciation, specifically the concentration of the free ion that is traditionally considered to be the most bioavailable (on a dissolved basis) and toxic form (Morel, 1983; Sunda and Guillard, 1976; Eriksen et al., 2001). While TSS was not a factor in this study due to filtration of samples prior to exposure, a positive correlation was observed between DOC and *M. galloprovincialis* EC50. This is worthy of note considering the relatively narrow range in DOC concentrations that were measured in San Diego Bay. The relationship between *M. galloprovincialis* embryo-larval development EC50s and DOC concentration is quite evident for DOC concentrations ranging from <1 to 12 mg L⁻¹ (Arnold et al., 2006), and it

is interesting to see this relationship here among so few samples and at relatively low DOC concentrations.

4.2 Whole body residue-effect thresholds

The utility of the critical body residue (CBR) approach for Cu was explored in this study by expressing toxicity in terms of whole body concentrations. Whole body ER50s varied little among experiments, as noted by very low CVs and overlapping confidence intervals, suggesting that whole body residues were a better predictor of toxicity than exposure water concentration. This is not unexpected, as tissue measurements reflect only the fraction of Cu available for uptake while dissolved water concentrations do not. CBRs, therefore, have been suggested to be a more appropriate indicator of adverse effects in aquatic biota than external water concentrations (McCarty and Mackay, 1993). Here, the apparent value of the CBR approach for Cu in *M. galloprovincialis* and *S. purpuratus* larvae is illustrated by very similar toxicity thresholds; a) among samples differing in water quality characteristics; b) between the two species in this study; and c) for other species and life stages, as described below.

The mean ER50s of 49.2 and 142 $\mu\text{g g}^{-1}\text{ dw}$, for *M. galloprovincialis* and *S. purpuratus*, respectively, are similar to literature values reported for similar species and life stages. A whole body Cu ER50 of 114 $\mu\text{g g}^{-1}\text{ dw}$ was calculated from data reported by Radenac et al. (2001) for comparable exposures with embryos of the European sea urchin (*Paracentrotus lividus*). This is particularly interesting because embryogenesis in *P. lividus* (mean EC50 = 62.5 $\mu\text{g dissolved Cu L}^{-1}$; Warnau et al., 1996; His et al., 1999; Fernandez and Beiras,

2001; Radenac et al., 2001; Lorenzo et al., 2002) appears to be considerably less sensitive to Cu than in *S. purpuratus* (species mean acute value = 12.8 µg dissolved Cu L⁻¹; USEPA, 2003) based on water concentrations.

To the best of our knowledge, this is the first time that CBRs have been determined for bivalve larvae. Geffard et al. (2002a), however, observed no effects in 24 h old Pacific oyster (*Crassostrea gigas*) D-shaped larvae at whole body residues of ~20 µg g⁻¹ dw in sediment elutriate exposures, which is comparable to our NOERs for *M. galloprovincialis* (18.4 – 24.0 µg g⁻¹ dw; Table 2).

Although ER50s differed to some extent between *M. galloprovincialis* and *S. purpuratus*, NOERs were essentially identical, suggesting similarity in the thresholds for effects for these two species. The apparent differences in the sensitivity of this endpoint between the species based on ER50 and EC50s, however, may be associated with differences in the ability to regulate or store Cu (Luoma and Rainbow 2005). In this study, lower bioconcentration factors (BCFs) observed at sub-effect concentrations in *S. purpuratus* (1938 ml g⁻¹) suggest a lower rate of uptake and/or more efficient elimination as compared to *M. galloprovincialis* (4574 ml g⁻¹) larvae.

Among the criticisms of whole body CBRs as valuable toxicity metrics for metals are that they do not provide an explicit measure of accumulation at the site of action of toxicity (e.g., a particular organ, a membrane bound ion transport system, or enzyme system)(e.g. Vijver et al., 2004). Similarly, CBRs do not necessarily differentiate between accumulated

metal that is metabolically available and that which has been detoxified following uptake. Inorganic granules (McGeer et al., 2003; Vijver et al., 2004) and metallothionein-like proteins (Widdows and Donkin, 1992; Roesijadi et al., 1997; McGeer et al., 2003; Vijver et al., 2004), for example, allow many aquatic organisms to sequester, store, and detoxify high concentrations of metal without observable effects. Metallothioneins have been measured at elevated levels in mussel (Roesijadi et al., 1997; Geffard et al., 2002b), oyster (Geffard et al. 2002a; Damiens et al., 2006) and sea urchin (Nemer et al., 1984) larvae following metal exposure originating with embryos. The repeatability of observed effects in the current study at specific whole body concentrations, however, suggests that whole body concentrations are very good surrogates for what is internally available for toxicity, for these species and endpoints. The reason may be that the amount of intracellular Cu that is complexed by metallothionein is small in comparison to the total amount of Cu that has been accumulated. This could be a reflection of the kinetics of metallothionein induction by metals (e.g., Viarengo et al., 1985 Roesijadi and Klerks, 1989) and short duration of the exposures used in our laboratory investigations, as well as the absence of the specific organs (e.g., gill and digestive gland) that are present in adults and particularly well suited for the purpose of MT synthesis and detoxification of metals such as Cu (Viarengo et al., 1980). Alternatively, the amount of Cu that interacts at sensitive intracellular biotic ligands may be a relatively small fraction of the total Cu, but proportional to the total amount of Cu that has been accumulated by the larvae.

Interestingly, the CBRs observed for embryo-larval development of *M. galloprovincialis* are similar to thresholds reported to impact a number of other toxicity endpoints in adult

Mytilus sp. Martin (1979) reported a median lethal residue (LR50) of $59 \mu\text{g g}^{-1}$ dw in adult *M. edulis*. Mean sublethal responses (e.g. growth, scope for growth, filtration, reproduction, condition, and change in bioaccumulation) to several bivalve species from 41 different studies were 48.0 and $21.1 \mu\text{g g}^{-1}$ dw for effects and no effects residues (Salazar and Salazar, 2007). For *Mytilus sp.* studies specifically, these values were 82.9 and $24.7 \mu\text{g g}^{-1}$ dw, respectively. While there is substantial evidence that *Mytilus* larvae, juveniles, and adults differ in their sensitivity to Cu based on water concentrations (Widdows and Donkin, 1992), these differences appear to be minimal when expressed as CBRs. Therefore, as has been proposed with field transplants of adult mussels (Salazar and Salazar, 2007), our data suggest that assessment of tissue concentrations in *M. galloprovincialis* larvae may be a valuable means of predicting biological impacts at sites of concern. Because exposure in filtered laboratory water isn't necessarily indicative of exposure in the field (Luoma, 1995), however, validation of these laboratory data should be pursued through field studies.

Although the life stages used in this study are lecithotrophic, the larvae quickly become planktotrophic upon development to veliger and pluteus stages for *M. galloprovincialis* and *S. purpuratus*, respectively, presenting potential consequences for use of these CBRs in field exposures. The laboratory experiments conducted here used filtered ($0.45 \mu\text{m}$) seawater, but an increase in mussel larval weight was accompanied by the development of the larval shell late in the 48 h exposure (Figure 6). His et al. (1989) also reported growth of *M. galloprovincialis* larvae in the absence of feeding on phytoplankton in laboratory experiments, and suggested that the larvae were feeding on bacteria. It has also been

shown, however, that bivalve veliger larvae have the capacity to directly assimilate dissolved organic material (DOM) from water, allowing them to live well beyond the expected their endogenous energy supply (Manahan, 1982; Moran and Manahan, 2004; Baines et al., 2007;). The role that DOM uptake may play in Cu accumulation for these life stages, therefore, needs to be studied.

4.3 Background concentrations below NOER

Background larval residues following exposure to Bay samples in the absence of added Cu (controls) were substantially below both NOERs and ER50s derived in this study, providing an additional line of evidence (in addition to water-based site-specific criteria) that ambient Cu concentrations in San Diego Bay should not negatively impact mussel or sea urchin populations. More specifically, the mean NOERs (20.6 and 23.4 $\mu\text{g g}^{-1}\text{ dw}$; Table 2) were 3.5 and 6.4 times higher than mean residues observed for mussels (5.90 $\mu\text{g g}^{-1}\text{ dw}$) and sea urchins (3.63 $\mu\text{g g}^{-1}\text{ dw}$) from the control treatments, respectively. Mean background tissue concentrations for both *M. galloprovincialis* and *S. purpuratus* in these experiments closely resemble those reported from other laboratory studies using similar life stages and from natural zooplankton samples collected from various geographic locations. Geffard et al. (2002a) reported background Cu concentrations of 5.11 $\mu\text{g g}^{-1}\text{ dw}$ for *M. edulis* larvae following 48 h exposures in clean filtered seawater from the Bay of Arcachon, France, while concentrations in *P. lividus* pluteus larvae were 5.9 $\mu\text{g g}^{-1}\text{ dw}$ in controls using a synthetic seawater (Radenac et al., 2001). Whole body tissue Cu concentrations in *M. galloprovincialis* or *S. purpuratus* larvae, therefore, appear to be a

valuable indicator of Cu exposure at concentrations well below those required to result in abnormal larval development.

4.4 Soft tissue ER50

ER50 estimates for soft tissue residues were somewhat higher than those based on whole body residues for both *M. galloprovincialis* and *S. purpuratus* larvae. This is due to the relatively low concentrations measured in the larval shell of *M. galloprovincialis* or the skeletal rods in *S. purpuratus*. Ringwood (1991) also reported very low accumulation of Cd in the larval shells of *I. californicum*, and suggested that the minimal concentrations that were measured were probably a result of adsorption to the shell as opposed to deposition. Low concentrations in the larval shell in our laboratory experiments contrast with generally high concentrations of metals, compared to soft tissues, observed in the exoskeletons (a location for storage and detoxification of metals) of many invertebrates (Horowitz and Presley, 1977). This could be due to the very short presence, on the order of hours, that these structures existed prior to sampling, thus minimizing the ability for adsorption from the seawater as well as deposition into these structures.

Expressing ER50s as soft tissue reduced the difference in sensitivity between *M. galloprovincialis* and *S. purpuratus* larvae to within a factor of two. This was because of the relatively higher proportion between shell and whole body in the mussel larvae as compared to the skeletal rods and whole body for the sea urchin pluteus. Soft tissue ER50s for *M. galloprovincialis* rose more than those of *S. purpuratus* because of the relatively

large contribution of shell in the total mass of the 48 h old *M. galloprovincialis* larva compared to the skeletal rods in the 96 h old *S. purpuratus* larva.

4.5 Comparison to LA50 for fish

The BLM is based on the notion that mortality, or other adverse effects, occurs when the metal-biotic ligand complex reaches a critical concentration (Santore et al., 2001). The model predicts LC50s or EC50s using easily obtainable water quality parameters rather than using costly and time-consuming toxicity testing. For fish, the concept of the biotic ligand is generally applied to physiologically active receptor sites at the gill surface that are involved in ionoregulatory processes (DiToro et al., 2001). The Cu ER50 data described herein for *M. galloprovincialis* and *S. purpuratus* larvae are considerably higher than the median lethal accumulation (LA50) values developed by MacRae et al. (1999) for rainbow trout ($0.64 \mu\text{g g}^{-1} \text{ ww}$, or $10 \text{ nmol Cu g}^{-1} \text{ ww}$) which were used for calibration of the freshwater BLM (DiToro et al., 2001). This apparent discrepancy may be due to a few reasons. First, it is already well known that the bioconcentration of metals can vary greatly among different organisms (Luoma and Rainbow, 2005). Invertebrate zooplankton are known to accumulate metals to particularly high levels (Horowitz and Presley, 1977; Fowler, 1986; Rainbow and White, 1990). Fish, however, closely regulate internal concentrations, and therefore, typically possess lower concentrations than invertebrate zooplankton populations (Horowitz and Presley, 1977; Fowler, 1986).

Perhaps more importantly, however, is that the critical concentrations reported in this study are based on whole and soft body residues, while the fish LA50 is based on accumulation

at the specific site of toxic action. Therefore, until the site of toxic action is determined and measured in *M. galloprovincialis* and *S. purpuratus* larvae, meaningful comparisons are difficult. In the interim, it is noteworthy that Cu concentration at the site of toxicity appears to be proportional to whole body concentrations for these specific organisms and endpoints.

5. Conclusions

Based on results of this study, it is apparent that critical whole body residues are a better predictor of Cu toxicity than water concentrations during embryo-larval development of *M. galloprovincialis* and *S. purpuratus* in laboratory exposures. Whole body concentrations are not always good measures of a toxicologically relevant accumulation because they do not distinguish between accumulated metals that are sequestered and stored from those that are biologically available internally for toxicity. The repeatability among CBRs observed in this study for water samples differing in physicochemical characteristics, however, suggests that for these endpoints, tissue residues may provide data that are useful in predicting toxicity in monitoring and assessment studies. Except for their small size, these species and life stages are advantageous and relevant because they often drive the setting of ambient WQC (particularly *M. galloprovincialis*), they require short exposure times, and the role of dietary uptake is generally considered negligible. Therefore, these data might be useful in decision-making with respect to the regulation of Cu in saltwater, and should be relevant toward the development of a saltwater BLM, which can ultimately be used to predict site-specific metal toxicity. Because of the differences in characteristics among water bodies, these findings should be validated by comparisons with similar

experiments in samples from other water bodies that vary more widely in DOC concentration than San Diego Bay. The direct relevance of these data for assessments of field populations also should be investigated.

Acknowledgements

This study was funded by the U.S. Department of Defense's Environmental Security Technology Certification Program (ESTCP) under project # ER-0523. The authors thank Jennifer Thompson and Christa Zacharias for assistance in the laboratory. Valuable comments from Mike Salazar and two anonymous reviewers improved the quality of the manuscript and are appreciated.

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Table 1. Mean ($\pm$ 1 S.D.) water quality measurements from controls (e.g. no added copper) for each of three larval toxicity and bioaccumulation experiments conducted with surface water from North and South San Diego Bay, CA.

Sample ID	Experiment #	pH	D.O. ^a (mg L ⁻¹)	Temperature (°C)	Salinity (‰)	DOC ^b (mg L ⁻¹)	Cu ^c (µg L ⁻¹)
North	1	7.89 (0.08)	8.65 (0.98)	15.5 (0.11)	34.3 (0.16)	1.30	0.9
North	2	7.85 (0.19)	7.28 (0.26)	15.3 (0.21)	34.1 (0.16)	1.70	0.6
South	2	8.01 (0.19)	7.38 (0.47)	14.8 (0.10)	35.8 (0.08)	2.24	2.5
North	3	8.09 (0.02)	7.97 (0.06)	15.8 (0.32)	34.2 (0.10)	2.47	0.9
South	3	8.20 (0.02)	7.97 (0.03)	16.0 (0.29)	35.4 (0.13)	3.43	2.5

^aD.O. = dissolved oxygen

^bDOC = dissolved organic carbon

^cCu= dissolved copper

Table 2. Toxicity metrics, based on exposure water concentrations and whole body residues, from seawater samples from North or South San Diego Bay, CA spiked with copper. Toxicity endpoints were normal embryo-larval development of either the Mediterranean mussel (*Mytilus galloprovincialis*) or purple sea urchin (*Strongylocentrotus purpuratus*). Metrics include the no-observable-effect concentration (NOEC) and residue (NOER), and median effects concentration (EC50) and residue (ER50). C.L.=confidence limit. Mean control (no added copper) tissue residues are also shown.

Organism	Sample ID	Expt #	Water concentration ($\mu\text{g Cu L}^{-1}$)		Whole body residue ($\mu\text{g Cu g}^{-1} \text{ dw}$)		
			NOEC	EC50 (95% C.L.)	Controls	NOER	ER50 (95% C.L.)
Mussel	North	1	4.10	6.36 (6.22-6.49)	5.84	18.4	50.3 (48.4-52.2)
	North	2	5.34	8.68 (8.47-8.89)	2.29	24.0	44.0 (36.9-52.7)
	South	2	7.08	12.8 (12.6-13.0)	9.65	19.3	53.4 (50.8-56.0)
Sea Urchin	North	3	9.1	14.3 (13.8-14.9)	3.68	22.9	131 (108-155)
	South	3	14.1	20.6 (20.3-21.0)	3.57	23.8	153 (115-192)

Table 3. Comparison of median effect residue (ER50) based on copper content determined in the whole body, the larval shell, and the soft tissue (* indicates estimate) from short-term exposures of copper in North San Diego Bay seawater to embryos of Mediterranean mussels (*Mytilus galloprovincialis*) and purple sea urchins (*Strongylocentrotus purpuratus*), in relation to the median effect concentration (EC50).

Organism	EC50 ($\mu\text{g L}^{-1}$)	ER50 ($\mu\text{g g}^{-1}$ dw)		
		Whole body	Shell/Rods	Soft tissue*
Mussel	9.46	47.7	2.65	76.9
Sea urchin	14.2	117	1.30	137

Figure 1. Map of San Diego Bay, California and approximate location of two surface water sampling area used in this study. The open circle at the mouth of the bay represents “North” samples and the solid circle at the head of the bay represents “South” samples.

Figure 2. a) Photograph from inverted light microscope of 48 h old Mediterranean mussel (*Mytilus galloprovincialis*) larvae under normal conditions, and b) following 24 h digestion with NaOH; and c) photograph of 96 h old purple sea urchin (*Strongylocentrotus purpuratus*) pluteus larva under normal conditions, and d) following 24 h digestion with NaOH. Actual sizes are approximately 120 and 200 μm , for individual mussel and sea urchin larvae, respectively.

Figure 3. Copper dose responses from embryo-larval development tests with mussels (*Mytilus galloprovincialis*) and purple sea urchins (*Strongylocentrotus purpuratus*) expressed as water concentration or whole body residues.

Figure 4. A) Median effect concentrations (EC_{50}) and B) median effect residues (ER_{50}) relative to dissolved organic carbon concentration for *Mytilus galloprovincialis* larvae following 48 h embryo-larval development exposures to copper. Error bars represent 95% confidence limits.

Figure 5. Comparison of median effect concentrations (EC50) from copper exposures in Mediterranean mussel (*Mytilus galloprovincialis*) embryo-larval development tests using both normal larval development and dry tissue weight as toxicity endpoints.

Figure 6. Mean (± 1 sd) dry weight per larva for developing Mediterranean mussel (*Mytilus galloprovincialis*) embryos exposed to a sub-effect Cu concentration ($4.1 \mu\text{g Cu L}^{-1}$) added to seawater collected from North San Diego Bay for two separate experiments.

Figure 7. Comparison of larval Mediterranean mussel (*Mytilus galloprovincialis*) and purple sea urchin (*Strongylocentrotus purpuratus*) whole body residues following exposure to different concentrations of copper in sea water from two locations (North and South Bays) in San Diego Bay, California.