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COPPER ACCUMULATION AND TOXICITY IN ISOLATED CELLS FROM GILLS AND HEPATOPANCREAS OF THE BLUE CRAB (*CALLINECTES SAPIDUS*)

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Abstract—In the present study, we used fresh preparations of mixed-cell populations to evaluate accumulation and toxicity of dissolved copper (1–100 μM) in isolated cells from posterior gills and hepatopancreas of the blue crab (*Callinectes sapidus*). For both gill and hepatopancreatic cells, significant increases in copper accumulation were observed after exposure to 50 or 100 μM copper. In gill cells, a linear increase in copper accumulation was observed over time. In hepatopancreatic cells, a maximum level of copper accumulation was achieved after 1 h of exposure, remaining unchanged up to 6 h. After 6 h of exposure, copper content in gill cells was 6.6-fold higher than that in hepatopancreatic cells. In both cell types, copper accumulation always followed a linear relationship with copper concentration in the incubation medium. Significant decreases in cell viability were observed after exposure to either 10 μM copper (gill cells) or 100 μM copper (gill and hepatopancreatic cells). Furthermore, an exponential rise to maximum-type relationship was observed between copper accumulation and toxicity in gill cells. Altogether, these findings indicate that the premise behind the biotic ligand model (BLM) approach is verified in isolated cells from posterior gills of the blue crab (i.e., toxicity is driven by copper accumulation in the biotic ligand, the gill cell). Therefore, these cells can be used as a model for the development of an in vitro BLM version for marine conditions. Isolated cells from the hepatopancreas, however, could be used as a model to better understand the mechanism of copper tolerance at a cellular level in crustaceans.

Keywords—Biotic ligand model Blue crab Copper Gills Hepatopancreas

INTRODUCTION

Copper is an essential micronutrient and acts as a cofactor in multiple enzymatic processes, but it is potentially toxic to aquatic organism when present in excess in the water. Elevated copper concentrations in the coastal and marine environments predominantly occur because of human activities. This increases the exposure and potential toxicity of this metal to coastal and marine organisms, including crustaceans.

Previous studies have shown that copper accumulation and toxicity are highly variable, depending on the crustacean species and life stage examined [1–4]. The cellular events underlying these effects are not yet fully understood, but studies reported in the literature indicate adverse effects of copper on active and passive ion transports. Therefore, copper has been recognized as an osmoregulatory toxicant that induces ionoregulatory disturbances in aquatic organisms [5–11]. Information concerning routes of copper accumulation and the mechanism of copper toxicity in brackish or marine invertebrates, however, is still scarce.

A long-term exposure to copper results in uptake and binding of copper ions to molecular sites of aquatic organisms [12,13]. Because gills are in contact with waterborne copper, these organs are the first target for metal toxicity [14,15]. In turn, hepatopancreas is a central organ involved in sequestration and detoxification of heavy metals [16]. Considering that the hepatopancreatic cell is the main site of accumulation, metabolism, and toxicity of metals like copper, isolated and cultured cells from gills and hepatopancreas could be a powerful tool for in vitro studies to gain a better understanding of these processes. In fact, conditions of in vivo exposure to aquatic contaminants can be reproduced under in vitro conditions using both fish and mollusks gill cells, as well as fish hepatocytes, and crustacean and mollusk hepatopancreatic cells [17–21]. Although the relevance of the systematic effects on toxicity and toxicant metabolism cannot be reflected from the in vitro results, cell culture allows rapid and relatively inexpensive testing. Furthermore, it often provides relevant information regarding the mechanism of toxicity.

In light of the above, the main goal of the present study was to use fresh preparations of mixed-cell populations to evaluate the toxicity of dissolved copper to, and its accumulation in, isolated cells from the gills and hepatopancreas of the blue crab (*Callinectes sapidus*).

MATERIALS AND METHODS

Animals

Male crabs (*C. sapidus*) were captured at the Patos Lagoon estuary (Rio Grande, RS, Southern Brazil), transferred to the laboratory, and acclimated to continuously aerated seawater at a salinity of 30 ppt (800 mOsmol/kg) for at least one week. During acclimation and tests, temperature and photoperiod were fixed at 20°C and 12:12-h light:dark, respectively. Crabs were fed to satiation twice a week with sliced fish. After acclimation, crabs were cryoanesthetized and killed by removal of the exoskeleton, and their posterior gills and hepatopancreas were dissected.

Primary culture and cell preparation

General procedures to obtain isolated cells from posterior gills of *C. sapidus* were performed according to the method described by Kelly et al. [22] with modifications. Briefly, the

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posterior gills from three crabs were excised and washed in sterile, Ca-free phosphate-buffered saline (PBS) with the following composition: 342 mM NaCl, 20 mM Na_2HPO_4, 1.7 mM K_2HPO_4, and 16 mM KCl (pH 7.6; 780 mOsmol/kg). Hemolymph cells were flushed out by perfusing the gills with PBS. Before cell dissociation, each gill was perfused with PBS containing 0.05% trypsin (Gibco®, Invitrogen). Tissues were minced into small pieces, placed in a beaker containing 20 ml of PBS with 0.035% trypsin and 0.04% chitinase (Sigma-Aldrich), and incubated in a shaking water bath (30°C) for 30 min. The resulting cell suspension was filtered (50-μm-mesh nylon filter) to remove nondissociated tissue and large debris and then mixed with a fresh PBS solution containing 10% fetal bovine serum (Gibco, Invitrogen) to stop trypsin activity. The filtrate was centrifuged (Model 204-N; Fanem) at 180 *g* for 10 min. The cell pellet was resuspended in culture medium (pH 7.6; 726 mOsmol/kg) prepared by adding 4.9 mM $NaHCO_3$, 8.1 mM KCl, 205 mM NaCl, and 5% fetal bovine serum to the 199 medium (Sigma-Aldrich).

Procedures to obtain isolated cells from hepatopancreas were carried out following the method described by Faucet et al. [23] with modifications. Hepatopancreas from three crabs was dissected, pooled, washed, and incubated (20 min) in PBS containing 10 mM Hepes (PBS-Hepes; pH 7.6; 790 mOsmol/kg), antibiotics (4% penicillin-streptomycin; Gibco, Invitrogen), and fungicide (1% fungizone; Gibco, Invitrogen). After incubation, the hepatopancreas was transferred to a new flask and rinsed twice with a freshly prepared PBS-Hepes solution without antibiotics and fungicide. It was sliced into small pieces with a razor blade and shaken at 80 rpm (Certomat-MO-II; Sartorius Stedim Biotech) for 30 min at room temperature (20°C). Dissociated cells were filtered (100-μm-mesh filter). The filtrate was collected in a 15-ml plastic tube and centrifuged at 90 *g* for 3 min (Model 204-N). The pellet containing cells was resuspended in PBS-Hepes. These procedures were repeated three times to wash cells. After the last wash, the pellet was resuspended in the same culture medium used for the gill cells, as described above.

Cell counting and viability in gill and hepatopancreas preparations were determined by the trypan blue exclusion method (0.08% trypan blue; Sigma-Aldrich). Cell density in primary cultures was adjusted to 5×10^6 cells/ml. Final volumes of cell preparations were in the range of 0.4 to 0.6 and 0.8 to 1.2 ml for gill and hepatopancreatic cells, respectively.

Copper accumulation

One aliquot (80 μl) of culture containing gill or hepatopancreatic cells was transferred into one well of a 96-well plate using a micropipette. Each well of the plate already contained 320 μl of a fresh culture medium prepared as described above, but with copper as $CuCl_2$ (Merck). Therefore, cells were tested at a final density of 10^6 cells/ml. Because fetal bovine serum seems to increase copper toxicity by binding copper and increasing its uptake by the cell [24], it was not added to the incubation medium. Copper was added 3 h before the beginning of the experiment to allow copper to equilibrate with the culture medium. Copper concentration in the exposure medium was analyzed by atomic absorption spectrophotometry (AAS 932 Avanta Plus; GBC Scientific Equipment). For each cell culture preparation, five replicates were used for each copper concentration and the control (no copper addition to the exposure medium). Measured copper concentrations were 1, 10, 50, and 100 μM. All procedures were performed in triplicate (i.e., in three different cell culture preparations).

At the beginning and after 1, 3, and 6 h of copper exposure, the exposure medium containing cells was gently mixed using a micropipette, transferred to a 1.5-ml plastic tube, and centrifuged at 1,200 *g* for 10 min (Hettich Zentrifugen, Model Mikro 22 R; Global Medical Instrumentation). For cell washing, the supernatant was discarded, and the pellet was resuspended with PBS (gill cells) or PBS-Hepes (hepatopancreatic cells). Centrifugation and washing procedures were repeated. After the second washing, cell samples were dried (60°C for 24 h) and digested with 50 μl of HNO_3 (Suprapur® Merck). Copper concentration in the samples was determined by atomic absorption spectrophotometry (AAS 932 Avanta Plus) after sample dilution with Milli-Q water (Milli-Corp). Copper concentration was expressed as μg Cu/10^6 cells.

Copper toxicity

Copper (1, 10, and 100 μM Cu) was added to the culture medium as $CuCl_2$ and equilibrated for 3 h. Gill and hepatopancreatic cells were then added to the culture medium without (control) or with copper at a final density of 10^4 cells/ml. Test media containing cells were placed in a 96-well plate, which was kept in an incubator (20°C) over the exposure time. At the beginning and after 1, 3, and 6 h of exposure, three samples from each well were collected, and cell viability was determined using the trypan blue exclusion method. Total and viable cells were counted using a hemocytometer (Neubauer chamber; depth, 0.1 mm). Cell viability was expressed as a percentage, considering the total number of cells in the respective control to be 100%. Control (no copper addition) and copper treatments were tested in triplicate for the same primary cell culture preparation. All procedures were replicated for six different primary cell cultures for each organ.

Statistical analyses

All data are expressed as the mean ± standard error. Copper accumulation and cell viability data were subjected to analysis of variance followed by the Tukey's test. Data normality and homogeneity of variances were checked previously. Mathematical data transformations were performed before statistical analysis of copper accumulation (log-transformation) and cell viability ($\sqrt{\text{arcsine-transformation}}$) data. In all cases, the significance level adopted was 95% ($p < 0.05$).

The relationship between copper accumulation and copper concentration in the incubation medium was determined by linear-regression analysis. The relationship between copper accumulation and gill cell mortality was determined by nonlinear-regression analysis (exponential rise to maximum).

RESULTS

Copper accumulation

At the beginning of the exposure period, copper concentrations in gill cells (11.75 ± 2.27 ng Cu/10^6 cells) was 3.0-fold higher than those in hepatopancreatic cells (3.91 ± 0.69 ng Cu/10^6 cells). In control cells, no significant change in copper concentration was observed over the exposure time for both gill and hepatopancreatic cells. The copper concentration was 11.54 ± 2.24 and 3.65 ± 0.67 ng Cu/10^6 cells in gill and hepatopancreatic cells, respectively.

Copper accumulation was measured in gill and hepatopancreatic cells over 6 h of exposure to different copper concentrations (1, 10, 50, and 100 μM). For both cells types, copper

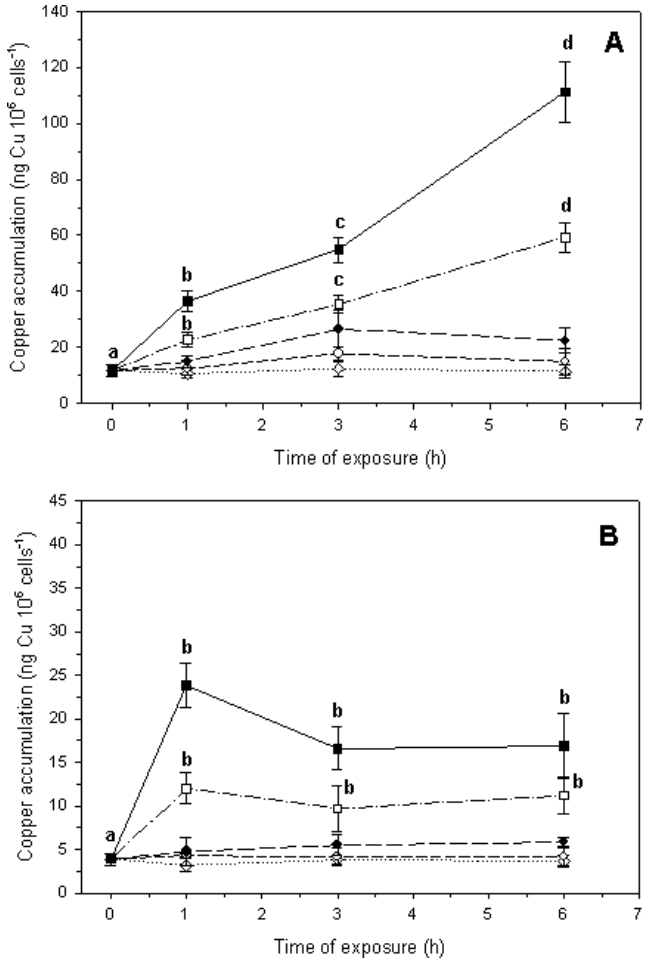

Fig. 1. Copper accumulation in isolated cells from posterior gills (**A**) and hepatopancreas (**B**) of the blue crab (*Callinectes sapidus*) exposed to 0 (◇), 1 (○), 10 (●), 50 (□), or 100 (■) μM copper ($CuCl_2$) for up to 6 h. Data are expressed as the mean ± standard error of three different cell preparations. Different letters indicate that the means are significantly different for cells exposed to 50 or 100 μM copper ($p < 0.05$). No significant differences were observed in the control or in cells exposed to 1 or 10 μM copper ($p > 0.05$).

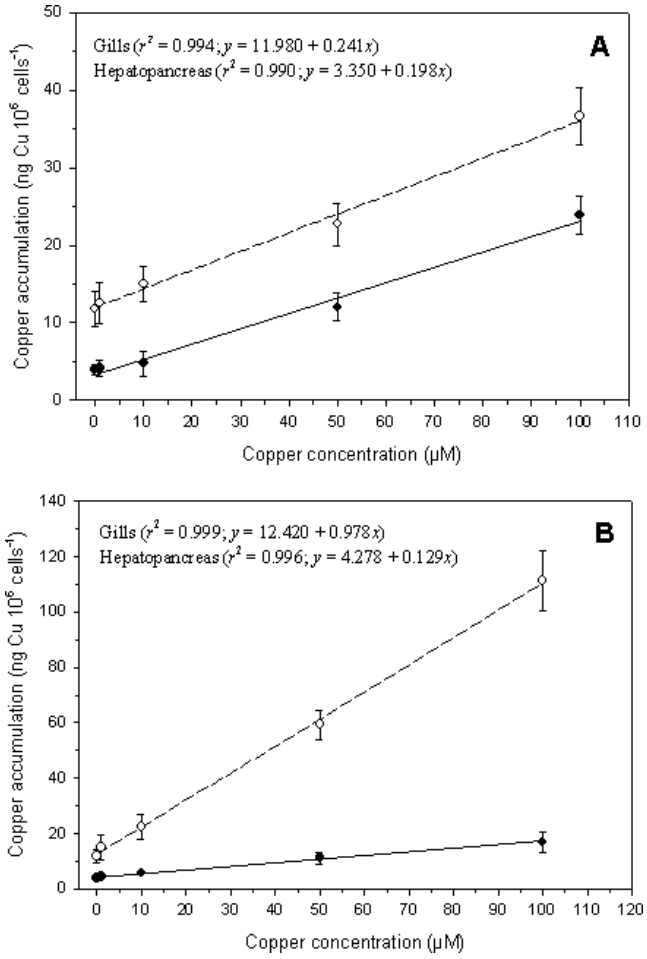

Fig. 2. Copper accumulation in isolated cells from gill (○) and hepatopancreas (●) of the blue crab (*Callinectes sapidus*) exposed to 1 h (**A**) or 6 h (**B**) to different copper ($CuCl_2$) concentrations. Data are expressed as the mean ± standard error of three different cell preparations.

content did not significantly change after exposure to the lower copper concentrations tested (1 and 10 μM). A significant increase, however, was observed after exposure to the higher copper concentrations (50 and 100 μM). After 6 h of exposure to 100 μM, the increase in cell copper content was higher in gill (9.5-fold) (Fig. 1A) than in hepatopancreas (4.3-fold) (Fig. 1B). Furthermore, copper accumulation kinetics were completely different between the two cell types. Gill cells showed a significant linear increase in copper accumulation over time (Fig. 1A), whereas hepatopancreatic cells showed a saturation-type kinetics. These cells were saturated with copper after 1 h of exposure and remained saturated for up to 6 h of exposure (Fig. 1B). Both gill (Fig. 2A) and hepatopancreatic (Fig. 2B) cells showed a linear increase in copper accumulation as a function of copper concentration in the exposure medium at both 1 and 6 h of exposure.

Copper toxicity

At the beginning of the exposure period, cell viability corresponded to 78.8% ± 2.5% in gill cells and 87.5% ± 0.8% in hepatopancreatic cells. Viability of both gill and hepatopancreatic cells did not change significantly over the exposure period under control conditions. The cell viability observed was 79.5% ± 1.3% and 87.6% ± 0.7%, respectively. It is important to note that no significant changes were observed in the total number of cells at the end of the exposure period (6 h) when compared to that measured at the beginning of the exposure period (data not shown).

Exposure to the lowest copper concentration (1 μM) did not induce a significant decrease in gill cell viability. Alternatively, a significant decrease in cell viability was observed after incubation with higher copper concentrations (10 or 100 μM). A 21.6, 28.5, and 31.0% decrease in cell viability was observed after 1, 3, and 6 h, respectively, of exposure to 10 μM copper. A 36.9, 38.8, and 44.1% decrease in cell viability was observed after 1, 3, and 6 h, respectively, of exposure to 100 μM copper (Fig. 3A). In hepatopancreatic cells, incubation with 1 or 10 μM copper did not induce any significant change in cell viability over the exposure period. Significant reduction in cell viability was only observed after exposure to 100 μM copper. In this case, maximum mean reduction in cell viability was only 14.6%. Furthermore, mortality was not dependent on the time of exposure (Fig. 3B).

Copper accumulation versus toxicity

Mortality of gill cells was clearly dependent on the cell copper content. Cell mortality markedly increased up to ap-

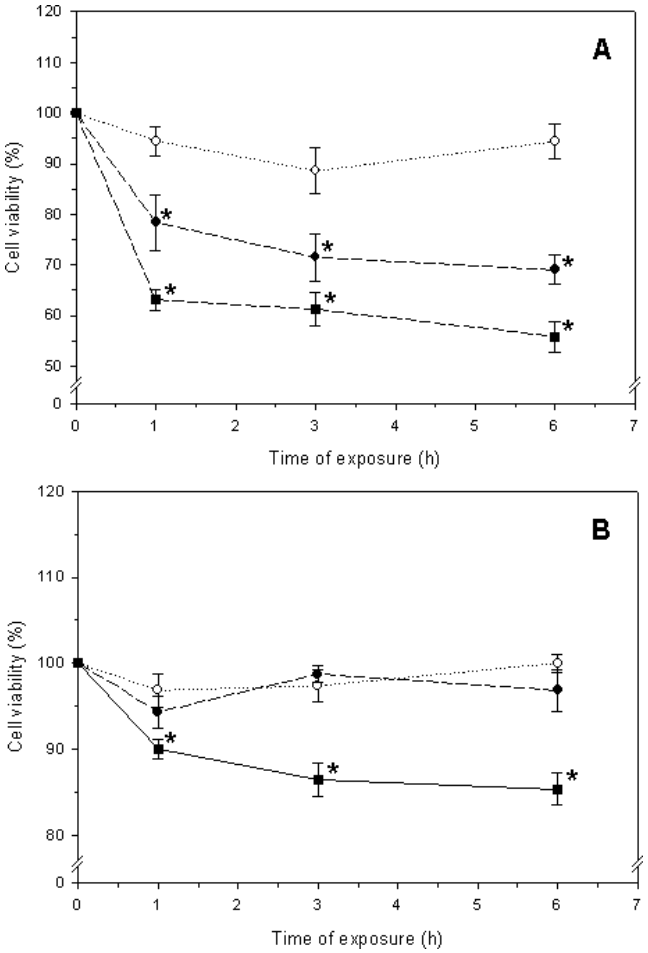

Fig. 3. Viability of isolated cells from posterior gills (**A**) and hepatopancreas (**B**) of the blue crab (*Callinectes sapidus*) exposed to different copper ($CuCl_2$) concentrations (○ = 1 μM; ● = 10 μM; ■ = 100 μM) for up to 6 h. Control values are normalized to 100% for each time point. Data are expressed as the mean ± standard error of six different cell preparations. Asterisks (*) indicates a mean value significantly different ($p < 0.05$) from the control.

proximately 40% as copper accumulation augmented up to approximately 40 ng Cu/10^6 cells. Higher copper levels in the gill cells did not induce any further significant cell mortality. In hepatopancreatic cells, any significant relationship was observed between cell copper accumulation and mortality in the range of the copper concentrations tested (Fig. 4).

DISCUSSION

In the present study, isolated cells from posterior gills and hepatopancreas of the blue crab (*C. sapidus*) were used to evaluate the potential accumulation and toxicity of dissolved copper. Data reported in the present study clearly indicate that procedures adopted for tissue dissection, washing, and incubation were quite adequate to obtain fresh preparations of isolated cells from gills and hepatopancreas of the blue crab. Therefore, procedures described in the present study could be adopted to obtain preparations of fresh isolated cells from gills and hepatopancreas of other crustaceans acclimated to seawater for many purposes, including short-term toxicological studies to access the possible different mechanisms involved in copper accumulation and toxicity at a cellular level in different tissues. This statement is based on the following facts: First, isolated gill and hepatopancreatic cells always showed viability values of higher than 75% for up to 6 h under control conditions (no copper addition to the experimental medium). Second, it was already possible to detect a significant copper accumulation in both gill and hepatopancreatic cells after 1 h of exposure to 50 or 100 μM copper. Third, it was possible to detect a significant copper toxicity in gill and hepatopancreatic cells after only 1 h of exposure to 10 or 100 μM copper. Fourth, different kinetics of copper accumulation and sensitivity to metal exposure were observed in gill and hepatopancreatic cells.

Marine invertebrates can take up toxic metals like copper across the integument via the gills when in contact with dissolved copper or through the gut after food ingestion. In turn, metal excretion may occur across the same tissues [7,16,25]. Because the gill epithelium is in direct contact with copper dissolved in the surrounding water, it is considered to be the main route of copper uptake from the dissolved phase. Consequently, it also is considered to be the first target for copper toxicity [14,15].

Data obtained in the present study clearly indicate that gill cells are able to regulate their copper levels in the presence of relatively low levels of copper (1 μM) in the surrounding medium. At very high levels of copper (100 μM), however, the mechanisms of copper extrusion from the cell are completely overwhelmed by those of copper uptake. At this point, it is interesting to note that once the cell capacity to regulate copper concentration is overwhelmed, as observed at 100 μM, copper toxicity is significantly expressed. In fact, time-dependent copper accumulation and toxicity were observed in isolated gill cells. It also is worth noting that a very significant correlation was observed between copper accumulation and toxicity in isolated cells from posterior gills of the blue crab. This finding is in complete agreement with the premise behind the biotic ligand model (BLM) approach [14,15]. According to this model, copper toxicity is a direct function of the amount of copper accumulated on or at the sites of toxicity at the gills of aquatic animals. Therefore, we suggest that isolated gill cells from the posterior gills of the blue crab can be used to better understand the mechanisms involved in copper accumulation and toxicity in marine crustaceans as well as to develop an in vitro BLM for copper to be applied in marine conditions. This last suggestion is based on the following facts: First, gills are the main route for copper entry and the first target for copper toxicity in aquatic animals [14,15]. Second, copper is considered to be an osmoregulatory toxicant [7,8,11]. Third, a straight linear relationship exists between copper concentration in the cell incubation medium and cell copper concentration. Fourth, an exponential relationship exists between cell copper burden and copper toxicity in isolated gill cells of the blue crab. Fifth, gill cells were markedly more sensitive to copper exposure than were the hepatopancreatic cells.

Alternatively, hepatopancreatic cells of the blue crab do not seem to be an adequate biotic ligand for the development of an in vitro BLM for marine conditions. Despite the fact that these cells also showed a linear increase in copper concentration as a function of the copper concentration in the incubation medium, no relationship was observed between cell copper burden and copper toxicity. It also is interesting to note that the maximum copper accumulation observed in the hepatopancreatic cells was achieved after only 1 h of exposure. This finding suggests that copper homeostasis after copper exposure is quickly reached in hepatopancreatic cells, whereas no copper homeostasis was observed in gill cells even after 6 h of exposure to 100 μM. Furthermore, copper toxicity was very low in hepatopancreatic cells

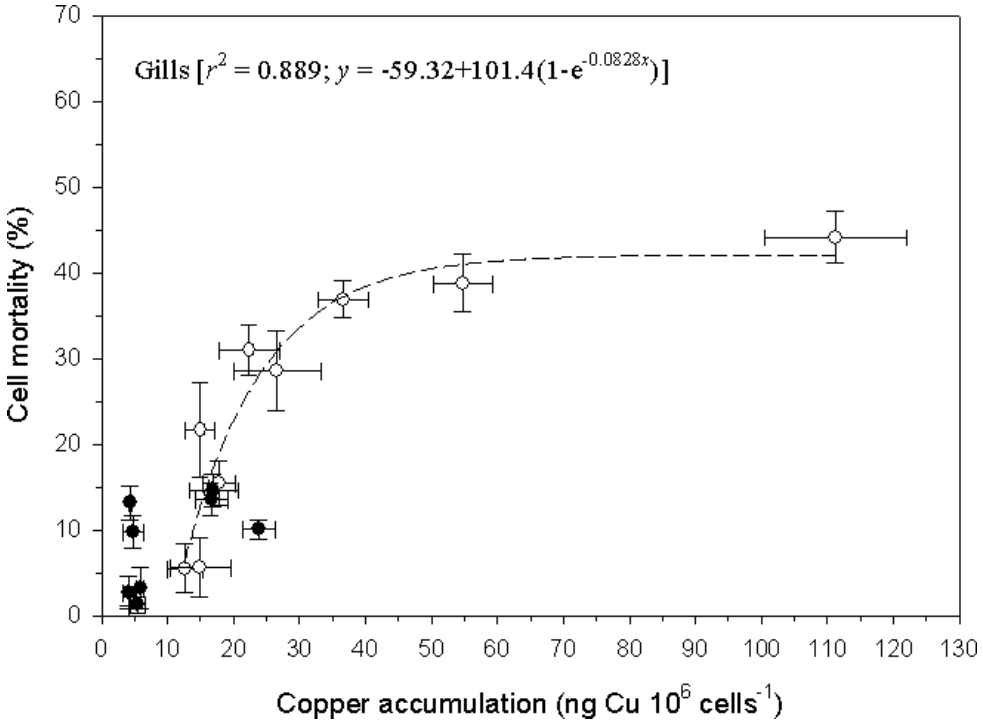

Fig. 4. Copper toxicity as a function of copper accumulation in gill (○) and hepatopancreatic (●) cells of the blue crab (*Callinectes sapidus*). Data are expressed as the mean ± standard error of three (copper accumulation) or six (copper toxicity) different cell preparations.

even after 6 h of exposure to 100 μM copper. Taken together, these data suggest that these cells could be used as an interesting model to investigate the mechanisms of copper uptake and extrusion, leading to a copper homeostasis at the cellular level, as well as to investigate the role of the hepatopancreas in tissue copper distribution in crustaceans.

A marked linear increase in copper toxicity as a function of copper concentration in the incubation medium was observed in gill cells in the range of 12 to 40 ng Cu/10^6 cells. After this level, no further toxicity was observed with increased cell copper burdens. This finding suggests that only one population of gill cells are being affected by copper exposure, with the remaining showing tolerance to the metal exposure. The epithelium of posterior gills in brachyuran crabs is composed by a monolayer of cells associated with the cuticle in each side of the ion-transporting gill lamella, where aggregates of ionocytes can be found. The pillar cells, however, are present in the gill epithelium, forming columns regularly disposed in the hemolymphatic space. This feature creates a series of lacunas that allows the hemolymph flow through the lamella [26,27]. Therefore, ionocytes are directly responsible for the ion transport across the gills and seem to be the preferred target for copper toxicity, because copper is recognized as an osmoregulatory toxicant that induces ionoregulatory disturbances in aquatic organisms [5–11]. Further studies are needed to confirm this hypothesis.

As discussed previously, copper cytotoxicity is clearly controlled by the level of copper accumulation in the gill cell. Therefore, the higher percentages of mortality observed in gill cells compared with in hepatopancreatic cells at the same exposure time and copper concentration can be explained by the higher copper accumulation in gill cells. This likely is associated with the low ability of gill cells to metabolize copper when compared to hepatopancreatic cells. It has been reported that the capacity to metabolize toxicants is much lower in fish gill cells than in fish hepatocytes [21,28]. Furthermore, copper appeared to have no effect on isolated digestive gland cells of the mollusk *Sepia officinalis* [19].

In hepatopancreatic cells, copper can be associated with metallothioneins or glutathione, transported into mitochondria, accumulated by lysosomes, transferred into the endoplasmic reticulum, or cross the basolateral cell membrane [16,17,29]. In crustaceans, the hepatopancreas has been recognized as a major organ of heavy metal homeostasis, sequestering and detoxifying dietary or waterborne metals. The hepatopancreas involvement in copper sequestration and accumulation may be more apparent after longer exposure to the metal. We need to consider, however, that copper is being sequestered, detoxified, and extruded continuously from the hepatopancreatic cells. Unfortunately, our data do not allow us to verify this hypothesis. Further studies are needed for a better understanding of this process at the cellular level. Gill cells seem to play a minor role in detoxification; however, they are in direct contact with waterborne toxicants and, as such, are considered to be a major route of uptake and a key organ for lethal damage by metals in particular [21,30]. As reported in the present study, gills rapidly accumulate copper following the onset of waterborne exposure, resulting in cytoxicity.

To summarize, data reported in the present study clearly indicate that isolated cells from the posterior gills of the blue crab are an adequate biotic ligand for copper in seawater, becoming an excellent model for the development of an in vitro BLM for marine conditions. Furthermore, isolated cells from the hepatopancreas could be used as a model to better understand the mechanism of uptake, detoxification, and extrusion of copper at a cellular level in crustaceans.

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