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Characterization of MXR activity in the sea anemone *Bunodosoma cangicum* exposed to copper



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ABSTRACT

Transmembrane proteins of the ABC family contribute to a multiple xenobiotic resistance (MXR) phenotype in cells, driving the extrusion of toxic substances. This phenotype promotes a high degree of protection against xenobiotics. The present study provides a better understanding of the MXR activity in the pedal disk cells of *Bunodosoma cangicum* exposed to copper, and further establishes the relationship between protein activity (measured by accumulation of rhodamine-B) and bioaccumulation of copper in these cells. Sea anemone cells were exposed for 24 h to copper (0, 7.8 and 15.6 µg/L) in presence and absence of MXR blocker (verapamil 50 µM). Results indicate that copper exposure increases intracellular metal content when ABC proteins were blocked, causing an increase in cellular death. The present study also verified the relationship between MXR activity, ATP depletion, and general metabolic activity (by MTT). MXR activity decreased in treatment groups exposed to copper concentrations of 15.6 µg/L and 10 mM energy depleting potassium cyanide. Metabolic activity increased in cells exposed to 7.8 µg Cu/L, but 15.6 µg Cu/L was similar to 0 and 7.8 µg/L. The presence of copper decreased the ABC proteins expression. The present study improves the knowledge of MXR in anemone cells and shows that this activity is closely associated with copper extrusion. Also, the copper exposure is able to modify the metabolic state and to lead to cytotoxicity when cells cannot defend themselves.

1. Introduction

The multiple xenobiotics resistance phenotype (MXR) is a capable of promoting cellular defense against exogenous substances and some endogenous metabolites, both *in vitro* and *in vivo* (Bard, 2000). This cellular resistance is expressed by a transmembrane super family of ABC transporter proteins (ATP binding cassette), which carry several xenobiotic substances out from the cells by active transport as a first line of cellular defense. The ABC proteins are well conserved throughout evolution and have already been identified in several groups of animals including sponges, cnidarians, mollusks, arthropods, fish and mammals (Epel, 1998; Bard, 2000; Kurelec et al., 2000; Ferreira et al., 2014). This capacity to extrude xenobiotic substances may confer different degrees of protection to animals living in contaminated areas (Kurelec et al., 2000).

One of the xenobiotic groups widespread in aquatic environments are the metals. Many studies have been performed to better understand how these substances affect cellular defense. Previous studies indicate

that metals may modify the activity/quantity of proteins associated with cellular MXR phenotype. Rocha and Souza (2012) observed a decrease in MXR activity in gills of *Corbicula fluminea* when exposed to lead, whereas Achard et al. (2004) observed an increase in ABC protein expression in bivalve gill cellular membrane when exposed to metals such as copper, cadmium, mercury, and zinc. However, the latter aforementioned authors did not analyze MXR activity. Della Torre et al. (2014) exposed *Mytilus galloprovincialis* to cadmium *in vivo* and *in vitro*. This study showed an increase in MXR activity and ABC gene expression when hemocytes and gill cells were exposed *in vitro*; however, *in vivo* exposure to cadmium resulted in an increase in gene expression without affecting MXR activity. Della Torre et al. (2012) demonstrated that MXR activity was inhibited in the PHLC-1 fish cell line (liver tumor cells) exposed to cadmium, mercury, arsenic, and chrome. Anjos et al. (2014) showed that MXR activity in sea anemone cells was variable when exposed to differing concentrations of copper *in vitro*. It is clear that the metal concentrations in experimental observations are important factors regarding MXR activity, gene expression and/or

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abundance of these proteins in the cellular membrane.

Metals have been described as a factor that may influence the MXR activity of cells. However, there is little knowledge about the relationship among bioaccumulation and MXR activity. Jeong et al. (2014) showed that metal concentrations (copper, cadmium, and zinc) increase in exposed copepod *Tigriopus japonicus* when MXR was inhibited. This study was the first relating metal accumulation and MXR activity in an invertebrate, showing that these proteins could be a pathway for metal extrusion: under inhibition, metal accumulation increased in the animal body. However, further studies are necessary to better elucidate the relationship between MXR and metal accumulation.

Bunodosoma cangicum is a sea anemone that is commonly found in Southern Brazil, inhabiting intertidal zones and strongly adhere to rocks or the inside of sandy crevices (Melo and Amaral, 2005). Sea anemones are susceptible to environmental contamination due to their low mobility. Genes of this superfamily ABC had already been identified in cnidarians (Venn et al., 2009). Moreover, Anjos et al. (2014) have shown that *B. cangicum* exhibited cellular defense response against copper exposure through an increase of MXR activity. Therefore, *B. cangicum* cells may display ABC proteins; the activity of those proteins could be different under copper exposure. However, the specific defense mechanism is not characterized for these cells. The present study elucidates aspects of MXR activity in cells of *Bunodosoma cangicum* exposed to copper and establishes the relationship between protein activity and bioaccumulation.

2. Materials and methods

2.1. Chemicals

All the salts used to prepare saline solution and PBS azide were from Sigma Aldrich (St. Louis, MO, USA), as well as the culture medium M199, verapamil hydrochloride, potassium cyanide, Rhodamine B and (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the secondary antibody anti-mouse immunoglobulin (Ig) G (tetra-rhodamine isothiocyanate) – TRITC. Copper chloride (CuCl_2 ; Sigma) was used to prepare treatments. The antibiotic/antimitotic was purchased from Gibco®, Invitrogen (Carlsbad, CA, USA). The monoclonal antibody C219 was from Signet-Covance® (Princeton, NJ, USA).

2.2. Collection and maintenance of anemones

Bunodosoma cangicum (Correa 1964) were manually collected in the intertidal area (32°09'40.25" S; 52°05'51.96" W) of Cassino Beach (Rio Grande, RS, Brazil). The animals were then transported to the laboratory in seawater and kept in glass aquariums at 20 °C temperature, constant aeration, photoperiod 12L:12D and salinity 30‰. The animals were fed once a week using small pieces of fish on the oral disk.

2.3. Extraction of sea anemone cells

Sea anemone cells were obtained through explant of pedal disk fragment generating primary culture as described by Anjos et al. (2014). The fragments were maintained in plate dish with 1 mL of culture medium M199 enriched with: (in mM) 41 NaHCO_3 , 80 KCl and 205 NaCl for adjustment of osmolality (approximately 800 mOsm kg/ H_2O , equivalent to coelenteron fluid) and 3% antibiotic/antimitotic (compilation: penicillin-streptomycin-fungizone). After 48 h in culture, the cells were removed, centrifuged and the pellet was resuspended in saline solution (in mM: 400 NaCl, 9 KCl, 9 CaCl_2 , 46 MgSO_4 , 2.2 NaHCO_3 , pH 7.4, approximately 800 mOsm kg/ H_2O). Density and viability of cells were analyzed via exclusion test with trypan blue 0.08%. All experiments were conducted with cells of viability above 80% and at a density of $2\text{--}3 \times 10^5$ cells/mL (200 μL for treatment).

2.4. Experimental conditions

Sea anemone cells were exposed to copper concentrations of 0 $\mu\text{g/L}$ (control, saline solution), 7.8 $\mu\text{g/L}$ and 15.6 $\mu\text{g/L}$ for 24 h. The 7.8 $\mu\text{g Cu/L}$ was defined with respect to Brazilian legislation (CONAMA resolution no. 357/2005), correspond to water quality criteria for Cu in sea water type II – intended for recreational fishing and recreational activities of secondary contact - and 15.6 $\mu\text{g Cu/L}$ was selected as a non-conforming concentration according to the Brazilian regulation. Same copper treatments were used in presence of ABC proteins blockers or decrease the energy available for the functionality of these proteins, including respectively:

Verapamil (VP) (50 μM - pilot experiments were performed with 30 and 50 μM , after those, the higher concentration was chosen) represents a well-known unspecific inhibitor of ABC proteins. VP acts as a calcium channel blocker and competes with other substrates as a competitive inhibitor (Srivalli and Lakshmi, 2012). A 1% Verapamil solution was prepared in ethanol and this solvent did not show any toxicity to sea anemones cells.

Potassium cyanide (KCN) 10 mM, is an ATP depletor that inhibits the electron transport chain (Watabe and Nakaki, 2007).

These experiments were conducted in independent days and animals (n represents one explant from one animal). Each experimental condition and exposures were performed in 96 wells micro plates or in 1.5 mL vials (for experiments of copper accumulation). All experiments were performed at least twice. Each chemical experiment (VP, KCN) was conducted with parallel experiments of cells exposed only to copper solutions (0, 7.8 and 15.6 $\mu\text{g/L}$), enabling results in absence of chemicals be grouped in the same MXR activity figures.

2.5. MXR analysis

MXR activity was measured based on cellular accumulation of rhodamine B (RB) which is a fluorescent substrate for ABC proteins (Kurelec et al., 2000). After exposure to copper in presence or absence of chemicals addition, cells were incubated in 10 μM Rhodamine B (RB) for 60 min, during this time the blocker or ATP depletor was kept. Then, cells were washed twice with saline solution to remove the substrate excess and transferred to 96 well microplate. Cells were visualized under an epifluorescence microscope (Olympus IX81) equipped with a fluorescence filter (WG) and a digital camera in order to capture images for posterior analysis (using free software Image J). The fluorescence exhibited in the images was measured and normalized by the number of cells present in the bright-field. For each treatment, 3 images from the same sample (each well) were captured and measured, and the mean of fluorescence intensity per cell was calculated. Cells with higher accumulation of RB indicate lower MXR activity because they could not remove the fluorescent substrate, while a lower accumulation indicates a higher MXR activity due to more efflux.

2.6. Cytotoxicity

In order to understand if blocking the ABC proteins promotes higher metal cytotoxicity, sea anemone cells were exposed to the different copper concentrations in presence or absence of VP. Two parameters were considered to measure the cellular toxicity: viability (cellular membrane integrity was analyzed after 24 h of copper exposure) and the number of viable cells, both evaluated via a trypan blue (0.08%) exclusion test.

2.7. Copper accumulation

In order to determine the effective contribution of ABC proteins to copper extrusion, ABC proteins were blocked and cellular copper content was quantified. Sea anemone cells ($2\text{--}3 \times 10^5$ cells/mL) were exposed to the different copper concentrations, for 24 h, in presence or

absence of VP. Following exposure, cells were washed and dehydrated for 24 h (50 °C), completely digested with 5 N HNO₃ and diluted with Milli-Q water. Copper concentration was measured via graphite atomizer atomic absorption spectroscopy (EAA Analyst 700, PerkinElmer), with detection limit of 0.0014 µg/L. The results obtained were normalized by the number of viable cells and expressed in µg Cu/10⁶ cell.

2.8. General metabolic activity

The general metabolism activity was measured using the MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Sea anemone cells (10⁶ cells/mL – higher number of cells were necessary in order to increase the absorbance signal) were exposed to copper solutions for 24 h, and cells were analyzed according to the specific protocol (Mosmann, 1983). This colorimetric MTT assay protocol measures the general metabolism of cells based on the cellular uptake of MTT and its subsequent reduction. Newer literature had shown that MTT could be converted in formazan not only in mitochondria but in other cellular compartments (Bernas and Dobrucki, 2002; Stockert et al., 2012). Higher metabolism activity results in higher formazan production. After copper exposure, cells were washed and incubated with MTT (500 µg/mL in saline solution) for 3 h. After that, the microplate was centrifuged (600 g for 5 min) and the supernatant was removed. Then, DMSO was added to dissolve the formazan crystals and the absorbance was recorded at 550 nm using EL808 (BioTek Instruments, USA). Results of MTT analysis were normalized by number of viable cells in each treatment (before MTT addition an aliquot was removed and counted).

2.9. ABC proteins expression

For the immunolabelling of ABC proteins, cells were exposed only to copper solutions as described before. After exposure, cells were washed to remove the contaminant and fixed in 4% formaldehyde in saline solution for 40 min. Then, cells were washed twice with phosphate buffer solution with added sodium azide (PBS; in mM: 340 NaCl, 12 Na₂HPO₄, 2.2, KH₂PO₄, 16 KCl and 5 EDTA, 10 NaN₃; pH 7.4, approximately ~800 mOsm kg/H₂O) and kept in refrigeration (4 °C) overnight. In sequence, membrane cells were permeated using Triton X-100 for 15 min, washed with PBS azide and serum fetal bovine was added for 4 h. After this, the primary monoclonal antibody C219 against ABC proteins (MDR1/ABCB1 and MDR3/ABCB3), was added and maintained overnight. Subsequently, cells were washed with PBS azide to remove the excess of antibody and added the secondary antibody rabbit anti-mouse immunoglobulin (Ig) G was added for 5 h. Rinsing was repeated and cells visualized in epifluorescence microscope with a fluorescence filter (WG). The capture and analysis of images were performed equally those explained to MXR analysis. High fluorescence means a high amount of proteins in the cells.

2.10. Statistical analysis

Results from general metabolic activity and immunocytochemistry were satisfactory for parametric assumptions and were analyzed by one-way ANOVA with Tukey test *post hoc*, the graphic shows mean and standard error (SE).

Experiments results from chemicals exposure were tested by parametric assumptions (data normality and homogeneity of variances; data were mathematically transformed when assumptions were not verified), and then, analyzed through ANOVA factorial type II, in order to determine if chemicals interacted with copper exposure. As no interaction was observed among experiments, the results were submitted to two ANOVA one-way, using *post hoc* Unequal N HDS (MXR analysis) or Tukey (other analysis). In order to compare the results between those cells exposed to copper with cells exposed to chemicals (VP or KCN), a *t*-test for independent samples was performed. Figures show the mean

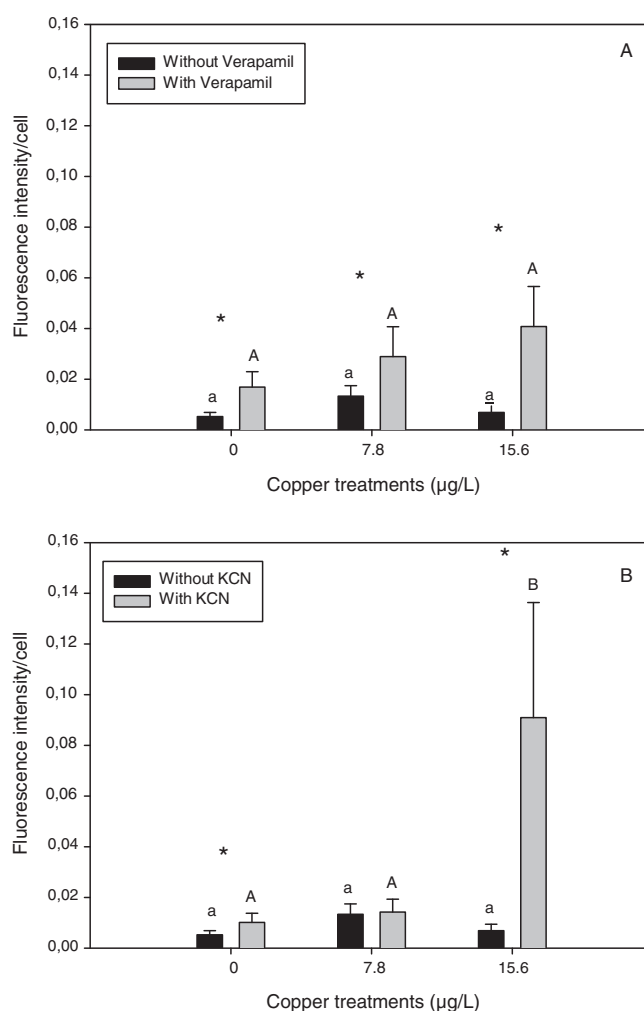


Fig. 1. MXR activity based on Rhodamine B accumulation in *B. cangicum* cells. Panel A: cells exposed to copper with/without verapamil (VP, n = 15–29). Panel B: cells exposed to copper with/without potassium cyanide (KCN, n = 11–29). Values represent means ± standard error. Lowercase letters indicate differences between treatments without chemical (only Cu); capital letters indicate differences between treatments with MXR inhibitor; * represents differences between same treatment without and with inhibitor.

and SE results of raw data. All the results assumed a significant difference when values of $p \leq 0.05$.

3. Results

3.1. MXR analysis

MXR activity was not altered under copper concentrations of 7.8 and 15.6 µg/L ($p = 0.43$) and it did not change even between copper exposures with VP ($p = 0.61$) (Fig. 1A). The results of *t*-test analysis showed the inhibition of MXR activity in presence of verapamil in all copper concentrations. In absence of copper (0 µg/L), the presence of VP increased the rhodamine accumulation three-fold when compared with cells without VP ($p < 0.001$). In cells exposed to 7.8 µg/L Cu, the presence of verapamil doubled the rhodamine accumulation ($p = 0.0006$) and those cells with verapamil at 15.6 µg Cu/L had an almost 6-fold increase of rhodamine B accumulation in when compared with cells in absence of blocker ($p < 0.001$).

Cells exposed to copper did not show any difference in MXR activity ($p = 0.43$) (Fig. 1B). However, in cells exposed to copper and the ATP depletor potassium cyanide, the MXR activity was smaller at

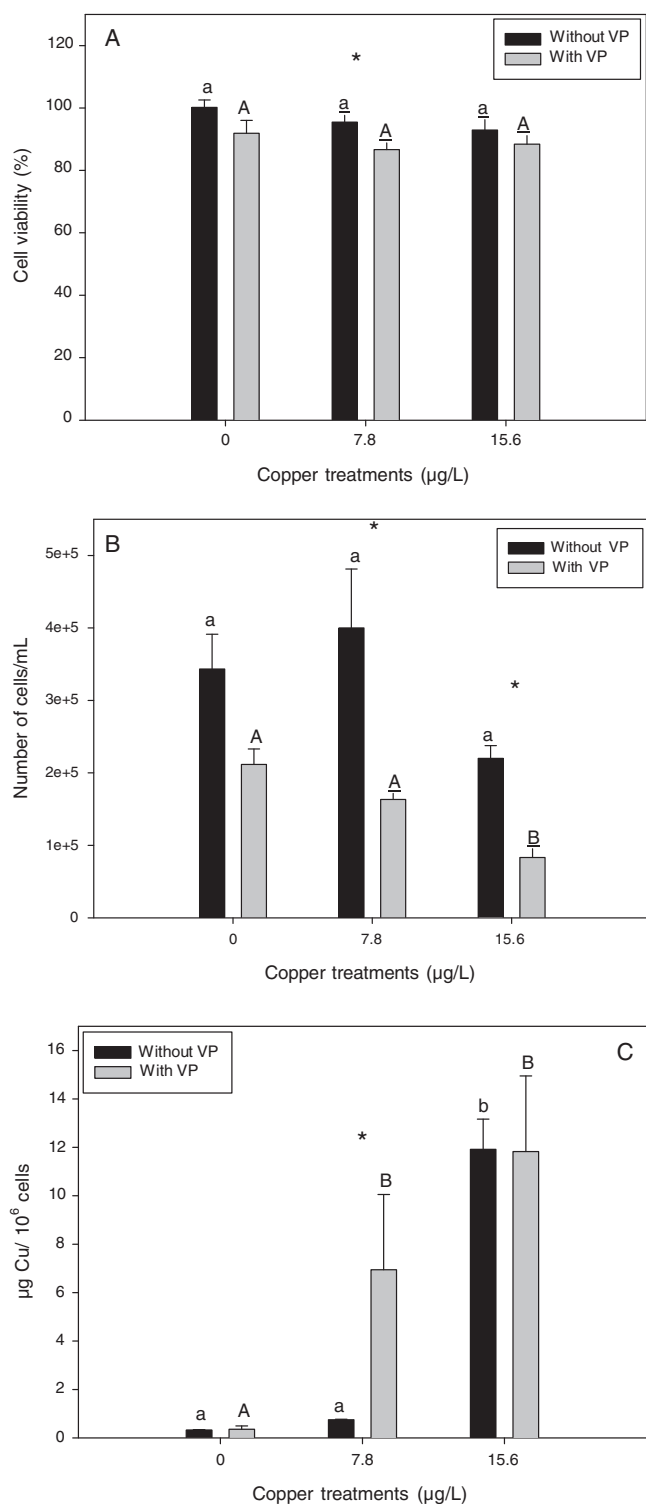


Fig. 2. Panel A: viability of cells of *B. cangicum* exposed to copper with/without verapamil ($n = 3$). Panel B: number of viable cells exposed to copper with/without verapamil ($n = 3$). Panel C: copper accumulation in cells exposed to copper with/without verapamil ($n = 4$). Values represent means \pm standard error. Lowercase letters indicate differences between treatments without VP (only Cu); capital letters indicate differences between treatments with VP; * represents differences between same treatment without and with VP.

15.6 $\mu\text{g Cu/L}$ treatment (increased 800% rhodamine B accumulation compared to control ($p = 0.015$)). Comparing results among absence/presence of KCN, results show that potassium cyanide inhibited MXR

activity in cells without copper and in 15.6 $\mu\text{g Cu/L}$ treatment (rhodamine B accumulated 2 and 15-fold, respectively).

3.2. Cytotoxicity

Fig. 2A shows sea anemone cells viability exposed to copper in presence or absence of verapamil and no statistical difference was observed. A decrease of cell viability (9%) was only observed in cells exposed to 7.8 $\mu\text{g/L Cu}$ with VP comparing to cells without blocker ($p = 0.02$).

Number of cells of *B. cangicum* exposed to copper in presence or absence of VP is shown in Fig. 2B. No statistical difference was observed in absence VP treatments, however among cells in presence of VP one difference was observed. Samples incubated with VP decreased the number of cells when exposed to 15.6 $\mu\text{g/L Cu}$ comparing to 0 $\mu\text{g/L Cu}$ + VP cells (decreased 60%, $p = 0.002$). Comparing the number of cells within each treatment, samples with copper and VP exposure showed fewer cells than those samples exposed to the same copper concentration without the blocker. The 15.6 $\mu\text{g Cu/L}$ in presence of VP treatment had a decrease of almost 60% in the number of cells compared to 0 $\mu\text{g/L Cu}$ in presence of VP treatment ($p = 0.03$ and $p = 0.003$ to 7.8 and 15.6 $\mu\text{g/L Cu}$ + VP, respectively).

3.3. Copper accumulation

Fig. 2C shows the copper content of *Bunodosoma cangicum* cells. Considering the cells exposed to copper (without verapamil), the metal accumulation increased in 15.6 $\mu\text{g Cu/L}$ (10-fold more than cells in 0 $\mu\text{g/L Cu}$; $p = 0.015$). In the presence of blocker, intracellular concentrations of copper were higher in both copper treatments when compared to control (0 $\mu\text{g/L Cu}$; $p = 0.003$).

In presence or absence of the blocker in copper treatments, cells exposed to 7.8 $\mu\text{g Cu/L}$ had a 14-fold increase of the intracellular Cu ($p < 0.0001$). The cells exposed to 15.6 $\mu\text{g Cu/L}$ had Cu increasing almost 2-fold when blocked with VP, however, it was not statistically different when compared to cells without VP ($p = 0.31$). Control cells (0 $\mu\text{g Cu/L}$) did not differ from cells incubated with verapamil ($p = 0.77$).

3.4. General metabolic activity

Metabolism was indirectly measured by MTT assay and the results are displayed in Fig. 3. Higher metabolic activity was observed in cells exposed to 7.8 $\mu\text{g/L Cu}$, while the lesser activity was observed in cells

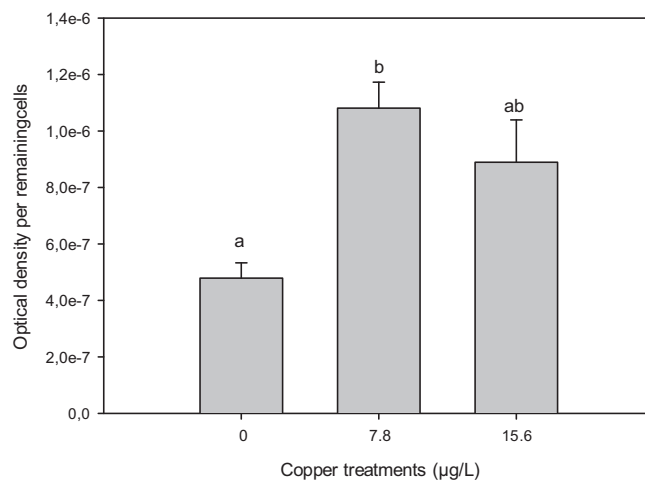


Fig. 3. General metabolic activity in *B. cangicum* cells exposed to copper, measured by MTT assay ($n = 4-5$). Values represent mean \pm standard error and different letters indicate differences between treatments.

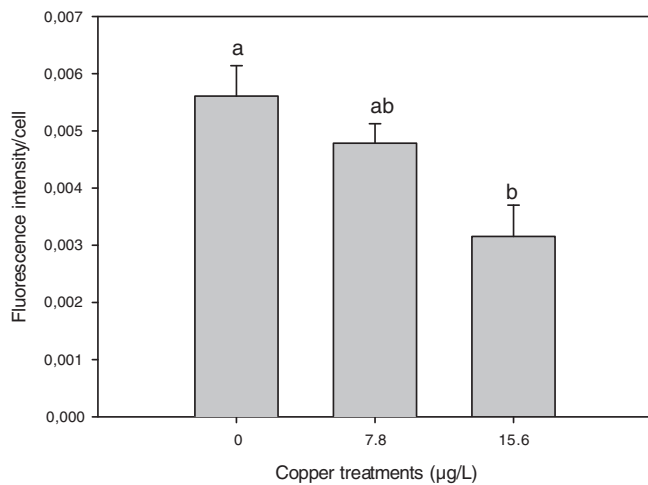


Fig. 4. ABC protein expression in *B. cangicum* cells exposed to copper ($n = 4$). The measurement was based on the secondary antibody fluorescence per number of cells. Values represent mean \pm standard error and different letters indicate differences between treatments.

exposed to control (0 µg Cu/L; $p = 0.017$). Results from 15.6 µg Cu/L exposure had metabolism between the other treatments, and it was not statistically different ($p = 0.07$ and 0.457 , respectively).

3.5. ABC proteins expression

Results of labeling cells using C219 are shown in Fig. 4. The 15.6 µg/L copper exposure decreased the amount of ABC protein in sea anemones cells compared with no exposed cells ($p = 0.014$). However, the amount of protein at 7.8 µg Cu/L exposure was not statistically different from 0 and 15.6 µg Cu/L ($p = 0.477$ and 0.093 , respectively).

4. Discussion

Transmembrane proteins of the ABC family contribute to the MXR phenotype in cells, leading the extrusion of toxic substances. MXR activity is commonly associated with high degree of protection against xenobiotics. However, literature showing whether this pathway is also responsible for extrusion of metals, correlating MXR and xenobiotic accumulation is scarce. The present study provides a better understanding of the MXR activity in cells of *Bunodosoma cangicum* exposed to copper, and establishes an association between protein activity and bioaccumulation of copper in these cells.

Sea anemone cell responses to copper exposure vary according to MXR activity. When the cells are exposed to different copper concentrations (7.8 and 15.6 µg Cu/L), no difference in the activity is observed. However, a tendency of inhibition at lower copper concentration and baseline activity (the same as 0 µg/L copper) at higher metal concentrations was observed. Anjos et al. (2014) observed a different result, where MXR activity increased in sea anemones cells exposed to 7.8 µg Cu/L, and returned to control activity levels at 15.6 µg Cu/L exposure. These results indicate differences in MXR activity of copper concentrations (7.8 and 15.6 µg/L). Although only two copper concentrations were evaluated, the authors believe that it might be possible that the 7.8 µg/L copper concentration represent a threshold concentration between activation and inhibition, thus varies, affecting MXR activity. Furthermore, the present study used a microscopy technique while the previous study (Anjos et al., 2014) used a fluorimetry technique. Microscopy involves observations of fluorescence inside of each cell while fluorimetry could produce noisier data, a fact corroborated by a study by Anjos et al. (2016) comparing both methods of MXR activity analysis. Therefore, the precise microscopy technique may have contributed to the differences observed in this study,

especially when the concentrations could induce variable activity.

MXR phenotype can be characterized by using ABC protein blockers and a fluorescent substrate, like rhodamine B. This characterization is largely used by adding verapamil (VP), a nonspecific inhibitor of ABC proteins, into the medium with cells. The VP inhibits substrate cell extrusion, resulting in higher fluorescence in cells incubated with the blocker (Kurelec et al., 2000; Shuilleabháin et al., 2005; Sandrine and Marc, 2007; Campos et al., 2014). In this study, the presence of the blocker inhibited the MXR activity in all copper concentrations when compared to treatments without VP (Fig. 1A). In order to verify if the blockade could increase bioaccumulation, the present study measured cellular metal content (Fig. 2C). When the MXR activity was compared in the presence and absence of VP, an increase in metal accumulation was observed in cells exposed to 7.8 µg Cu/L when compared to the same copper treatment without VP. These results indicate the presence of copper and the blocker decreasing cellular defense, whereby metal extrusion is inhibited due to blocking of ABC proteins by VP. Blockade cells exposed to 15.6 µg Cu/L did not show any difference in metal accumulation when compared to cells in the absence of the blocker. Fishes have a homeostatic regulation of copper uptake, reaching a steady state gradually after being exposed to metal for some time, where the time to achieve the plateau is variable to each specie (Marr et al., 1996; Taylor et al., 2000; Grosell and Wood, 2002). According to Marr et al. (1996), if the accumulation increases through exposure (time and concentration) and the accumulation capacity is regulated by depuration, the time to reach steady state will increase as the capacity of accumulation increases. Therefore, it is possible that sea anemones also have a similar accumulation dynamic and because the mechanism of copper extrusion has been overwhelmed and/or the uptake may have achieved its maximum, this way, the copper accumulation is similar in cells exposed to higher copper concentration, independent of MXR blockage.

Comparisons between xenobiotic accumulation and ABC protein activity are common in studies involving antitumor treatments, however, correlation with environmental scenarios are scarce. A relevant study to compare the results here is Jeong et al. (2014), who demonstrated that after inhibition of ABC proteins with verapamil, the xenobiotic accumulation increases. In their study, copepods were exposed to cadmium, copper, and zinc for 24 h, associated or not with a specific inhibitor. As a result, the animals incubated with the inhibitor and exposed to metals had higher metal accumulation (analyzed by fluorescence labeling intensity). Furthermore, copepod mortality increased in metal treatments (copper, cadmium, and zinc) with inhibited ABC proteins, allowing the authors to conclude that verapamil is a cellular inhibitor of metal efflux, resulting in high mortality.

In order to analyze the blockage of ABC proteins and copper cytotoxicity in *B. cangicum*, the viability of sea anemone cells exposed to copper in the presence or absence of verapamil was evaluated. In general, the viability of cells exposed to the different copper treatments in the presence or absence of verapamil is not modified, however the ABC protein blockage causes a decrease in cell viability in cells exposed to 7.8 µg Cu/L when compared to cells exposed to the same copper concentration but without verapamil (Fig. 2A). On the other hand, in the samples with 15.6 µg Cu/L and VP, the number of cells reduced significantly despite the similar viability. Anjos et al. (2014) showed that sea anemone cells exposed to copper 15.6 µg/L decreased the viability and number of cells due to cellular committal. The copper concentration used in their study was the same as that used here, and the difference found in viability could be explained by the method applied. Although the method was the same, the trypan blue exclusion method analyzes the membrane integrity and takes an immediate evaluation, and even small temporal differences between each analysis might tend to overestimate the cell viability (Freshney, 2010), thus to measure the cytotoxicity, it is recommended to use the number of cells as well. It is therefore clear that MXR activity contributes to the efflux of metals from cells and this extrusion is important to maintain the cells

healthy. It is observed that the ABC proteins blockade decreases the viability in cells exposed to 7.8 µg Cu/L when comparing the absence and presence of VP, besides, the number of cells decreases in both copper concentrations when co-exposed to verapamil. Therefore, the cytotoxicity increased when cells were exposed to verapamil combined with copper concentrations. Anjos et al. (2014) analyzed the cytotoxicity and MXR activity in 6 and 24 h. In the first 6 h, the copper damaged the cellular membrane but did not reduce the number of cells nor alter the MXR activity. After 24 h, the membrane integrity was debilitated and the copper led to cellular death, reducing the number of cells, similar to the findings here. They also found that when MXR activity was activated the number of cells did not reduce despite the viability decrease. Therefore, the extrusion of copper by MXR is effective in protecting the cells and the combined analysis of membrane integrity and number of cells is a better form of estimating the cytotoxicity.

Additionally, the results of general metabolic activity could be related to the viability and number of cells in the presence of copper (without VP). A higher metabolic activity was observed in 7.8 µg Cu/L, indicating that cells were spending energy to counter the toxic effects. This is effective, observing that the viability, number of cells and MXR activity remained similar to cells with 0 µg Cu/L. The increase in metabolic activity seems to deal with the stressor. However, 15.6 µg Cu/L samples showed a general metabolic activity between 0 and 7.8 µg Cu/L and indicated that although cells try to maintain their health, they could not handle more in this situation and the copper reduced the number of cells, i.e., cell death occurred. In the literature, MTT analysis is shown as a measurement for cellular viability (Rtal et al., 1996; Le Bihan et al., 2004; Vazzana et al., 2014). However, it may not be a good indicator since the MTT assay is related to general metabolic activity (Bernas and Dobrucki, 2002; Stockert et al., 2012), thus, results could over or underestimate the viability depending on the cells metabolic state.

MXR activity is an ATP-dependent mechanism that may be compromised in an ATP depletion state. An experimental ATP depletion is performed by using mitochondrial electron transport chain complex I and IV inhibitors such as potassium cyanide (Watabe and Nakaki, 2007). The aforementioned study observed that potassium cyanide decreased MXR activity after exposure to 15.6 µg Cu/L (Fig. 1B). Presumably, cellular extrusion at the highest concentration of copper exposure became too energetically expensive and was subsequently disrupted, whereas the lower copper concentration was controlled.

The results discussed above are unprecedented in current literature, and to the best of knowledge of the authors, it is the first time that metal content measurements in cnidarian cells are related to MXR activity and cytotoxicity. Moreover, the present study elucidates aspects of the energy system involving ABC proteins such as energy requirement and general metabolism activity.

The present study also evaluated the ABC protein expression in sea anemones cells. It is showed that the 15.6 µg Cu/L treatment decreased the ABC proteins expression in the cellular membrane of *B. cangicum*. Although other studies show that copper induces this ABC protein and genic expression (Achard et al., 2004; Venn et al., 2009), it is well known that copper is a transition metal that may increase reactive oxygen species (ROS) (Stohs and Bagchi, 1995; Gaetke and Chow, 2003; Sandrini et al., 2009; Main et al., 2010; Grosell, 2012). ROS can impair proteins, copper could also bind directly to protein amino acids, potentially leading to dysfunctionality (Gaetke and Chow, 2003; Roy et al., 2009; Grosell, 2012; Tamás et al., 2014). In the study of Anjos et al. (2014), it was observed that reactive oxygen species increase in sea anemones cells exposed to 7.8 µg Cu/L, and even more so to 15.6 µg Cu/L. Therefore, the hypothesis in this work that ROS is causing the impairment in ABC protein expression is supported. This dysfunctionality, caused by heavy metals in general (directly or indirectly), could be attributed to perturbation in protein folding, leading the proteins to an inactive state from an incorrect conformation (Sharma et al., 2008; Tamás et al., 2014). Cells had the ability to degrade

abnormally folded proteins, leading to their hydrolysis (Goldberg, 2003). Therefore, the decrease in ABC protein expression is possibly a result of processes combination: ROS increase, damage in ABC proteins and conformational state with subsequent hydrolysis.

In summary, MXR activity remained similar to control groups in all copper treatments, and cytotoxicity was avoided even with higher copper accumulation in cells exposed to 15.6 µg Cu/L, however, the copper toxicity impaired the ABC protein expression. These results indicate an effective cellular defense system in cells exposed to copper, mainly at 7.8 µg Cu/L, even without increasing the MXR activity. These cells defend themselves pumping the excess copper out and protecting against cytotoxicity. However, the cost of this adjustment was observed through an increase in metabolic activity. Furthermore, the MXR activity seems to be closely associated with copper extrusion, since cellular copper content and cytotoxicity increased when MXR activity was inhibited. The present study showed novel cellular response patterns to metal exposure, MXR activity, and protein expression, it also elucidates the cellular defense mechanisms of *Bunodosoma cangicum* after being exposed to copper.

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