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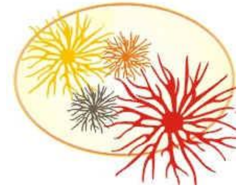

Células de anêmonas *Bunodosoma cangicum* expostas ao cobre: citotoxidade, defesa e dano de DNA

Biól. Vanessa Abelaira dos Anjos

Dissertação apresentada no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas- Fisiologia Animal Comparada, como parte dos requisitos para obtenção do título de MESTRE em Fisiologia Animal Comparada.

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Orientadora: Profª. Drª. Marta Marques de Souza

You live, you learn
You love, you learn
You cry, you learn
You lose, you learn
You bleed, you learn
You scream, you learn

(You learn – Alanis Morissette)

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Apresentação do Formato da Dissertação

O presente trabalho é constituído de uma *Introdução Geral*, que visa apresentar o tema a ser desenvolvido, tendo o propósito de fornecer embasamento teórico sobre o assunto. Em seguida são apresentados os *Objetivos Gerais* e *Específicos* da Dissertação. Os aspectos metodológicos, os resultados e a discussão dos mesmos estão apresentados na forma de um *Manuscrito* a ser submetido ao periódico **Toxicology *In vitro.*** Por fim são feitas *Considerações Finais* sobre o trabalho.

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Resumo

Anêmonas-do-mar são pólipos solitários, bentônicos, de pouca mobilidade, que habitam regiões entre-marés. Devido a estas características, são organismos que podem ser atingidos diretamente pela poluição aquática, no entanto, são pouco utilizados como modelo ecotoxicológico. O cobre é um metal essencial, que em altas concentrações pode ser tóxico, sendo bastante comum em ecossistemas marinhos. Um dos mecanismos de toxicidade do cobre envolve a produção de espécies reativas de oxigênio (ERO), podendo levar as células ao estresse oxidativo, que tem como característica danos celulares, inclusive no DNA. Muitos organismos possuem um mecanismo que bombeia os xenobióticos para fora da célula – *multixenobiotic resistance* (MXR) – que visa prevenir as células dos danos tóxicos causados pelo contaminante. Com isso, o presente trabalho estudou a capacidade de defesa e dano ao DNA à toxicidade causada pelo cobre em células de anêmonas *Bunodosoma cangicum.* Para isto, células de anêmonas, mantidas em cultura primária através de explante do disco podal, foram expostas ao cobre a duas concentrações (7,8 $\mu g.L^{-1}$ Cu e 15,6 $\mu g.L^{-1}$ Cu), além do grupo controle, por 6 e 24 h. Antes e após as exposições as células tiveram sua viabilidade avaliada através do método de exclusão por azul de tripan (0,08%) para analisar a citotoxicidade. Parâmetros como a indução do mecanismo MXR através do método de acúmulo de rodamina-B, espécies reativas de oxigênio e ensaio cometa, também foram avaliados. Os resultados obtidos mostram que o cobre é citotóxico, sendo constatada uma queda na viabilidade e no número de células, principalmente após 24 h de exposição, sendo que na concentração de cobre de 15,6 $\mu g.L^{-1}$, foi possível observar uma diminuição de 40% na viabilidade e uma redução em 36% no número de células ($p < 0,05$, $n = 6$). Em relação ao fenótipo MXR, foi observada uma ativação do mecanismo apenas naquelas células expostas ao cobre 7,8 $\mu g.L^{-1}$ (53%) no tempo de 24 h ($p < 0,05$, $n = 5$). Na análise da geração de ERO foi observado um aumento de 11,5% naquelas células expostas por 6 h na concentração mais alta de cobre 15,6 $\mu g.L^{-1}$. Nas células que foram expostas por 24 h, o aumento de espécies reativas pode ser percebido já na concentração de 7,8 $\mu g.L^{-1}$, elevando-se para cerca de 20% quando exposto a 15,6 $\mu g.L^{-1}$ ($p < 0,05$, $n = 4\text{-}5$). Quanto ao dano de DNA, foram vistas quebras na molécula desde 7,8 $\mu g.L^{-1}$ Cu em 6 h, com danos ainda mais salientes naquelas células expostas por 24 h, na concentração de 7,8 $\mu g.L^{-1}$ Cu ($p < 0,05$, $n = 3\text{-}4$), e para 15,6 $\mu g.L^{-1}$ Cu a viabilidade celular (número de células) não permitiu a análise. Com base nestes dados, pode-se dizer que o cobre, mesmo em baixas concentrações causa estresse em células de *B. cangicum*, sendo citotóxico. Este metal causa estresse oxidativo com dano à molécula de DNA mesmo com a ativação do mecanismo de defesa.

Palavras-chave: anêmona, cobre, citotoxicidade, MXR, ROS e dano ao DNA.

Abstract

Sea-anemones are solitary polyps, benthic, of low mobility, inhabiting intertidal regions. Due to these characteristics, they are organisms that can be directly affected by water pollution, however, are infrequently used as ecotoxicological models. Copper is an essential metal, which in high concentrations can be toxic, being quite common in marine ecosystems. One of the mechanisms of copper toxicity involves the production of reactive oxygen species (ROS), leading cells to oxidative stress. This stress can cause cell damage, including to DNA. Many organisms have a mechanism that pumps the xenobiotics out of the cell - multixenobiotic resistance (MXR) – which aims to prevent the cells from damage caused by toxic contaminant. So, the present work studied the ability of defense and DNA damage caused by copper toxicity in cells of *Bunodosoma cangicum* anemones. For this, exposure of anemones cells were held, kept in primary culture through pedal disk explant, to copper in two concentrations (7.8 µg L^{-1} Cu and 15.6 µg L^{-1} Cu), in addition to the control group, for 6 and 24 hours. Before and after exposure the cells had their viability assessed through the exclusion method by Tripan blue (0.08%) to analyze the cytotoxicity. Were also evaluated the induction of MXR mechanism through the method of rhodamine-B accumulation, reactive oxygen species and comet assay to assess the possible damage to the DNA molecule. The results obtained show that copper is cytotoxic, being observed a drop in viability and number of cells, especially after 24 hours of exposure, noticing that in copper concentration of 15.6 µg L^{-1}, it was possible to observe a 40% decrease in viability and a 36% reduction in the number of cells ($p < 0.05$, $n = 6$). In relation to the MXR phenotype, it was observed an activation of the mechanism only in those cells exposed to copper 7.8 µg L^{-1} (53%) for 24 hours ($p < 0.05$, $n = 5$). In the analysis of the generation of ROS it was observed an increase of 11.5% in those cells exposed for 6 hours in the highest concentration of copper 15.6 µg L^{-1}. In cells that have been exposed for 24 hours, the reactive species increase can be noticed already in the concentration of 7.8 µg L^{-1}, increasing to about 20% when exposed to 15.6 µg L^{-1} ($p < 0.05$, $n = 4$-5). As for the DNA damage, molecule breaks from 7.8 µg L^{-1} Cu for 6 hours were seen, with even more salient damage in those cells exposed for 24 hours, at a concentration of 7.8 µg L^{-1} Cu ($p < 0.05$, $n = 3$-4), and to 15.6 µg L^{-1} Cu the cell number was not enough for analysis. Based on these data, it can be said that copper, even at low concentrations cause stress in *Bunodosoma cangicum* cells, being cytotoxic. This metal activates cellular defense mechanisms, however, this defense seems to be time and concentration dependent, leading cell to an oxidative stress with subsequent damage to the DNA molecule.

Keywords: Anemone, copper, cytotoxicity, MXR, ROS and DNA damage.

Introdução geral

O filo Cnidaria é um grupo altamente diverso que inclui águas-vivas, as anêmonas-do-mar, os corais, as hidras, e algumas outras formas menos familiares, como hidróides e sifonóforos. São animais que possuem simetria primária radial, tentáculos, estruturas urticantes ou adesivas (cnidas), cavidade gastrovascular incompleta como única cavidade corpórea e uma mesogléia (camada intermediária). Os cnidários carecem de cefalização, o sistema nervoso é uma rede nervosa simples, composto por neurônios nus e não polares, também não possuem estruturas individualizadas para trocas gasosas, excreção ou circulação. Além disso, são os animais que possuem menos tipos celulares que todos os outros animais (exceto esponjas e mesozoários), possuindo menos tipos de células que um único órgão de muitos outros animais. Este filo data do Pré-Cambriano (605 milhões de anos atrás), e seus membros tiveram papéis importantes em vários cenários ecológicos no decorrer de sua longa história (Brusca & Brusca, 2007).

As anêmonas-do-mar são cnidários da Classe Anthozoa, Ordem Actiniaria, que apresentam apenas a forma polipóide em seu ciclo de vida. São organismos solitários, que se alimentam de pequenas presas presentes no plâncton, principalmente crustáceos devido a sua grande abundância, e também de algumas presas bentônicas móveis. Podem ainda absorver matéria orgânica dissolvida (especialmente importante para anêmonas pequenas e as que se enterram) e muitas vezes apresentam interações com organismos fotossintetizantes (zooxantelas ou zooclorelas) (de Capitani, 2007).

Estes animais são osmoconformadores (a osmolalidade do fluido corporal do animal varia conforme a osmolalidade do meio externo) bentônicos, de pouca mobilidade, que habitam desde regiões intermarés, recifes costeiros rasos, costões rochosos a ilhas oceânicas e atóis (Melo & Amaral, 2005). Normalmente, podem ser encontrados associados a rochas ou em locais protegidos tais como fendas ou parte inferior das rochas, mas em situação de baixa maré podem se encontrar emersas (Amaral *et al.*, 2000).

Bunodosoma cangicum Corrêa 1964 (Fig. 1) é uma anêmona-do-mar comumente encontrada na zona intermarés das regiões Sul e Sudeste do Brasil. Elas costumam estar em rochas ou em fendas arenosas, onde

permanecem enterradas, fixas, ou não, ao substrato rochoso, expondo somente o disco oral (Melo & Amaral, 2005). Além disso, estas anêmonas apresentam uma coloração parda que as permite camuflar-se facilmente no substrato onde vivem, principalmente quando se encontram encolhidas (Ribeiro-Costa & Rocha, 2006). Quando expostas, permanecem completamente fechadas evitando a dessecação. As vesículas encontradas em sua coluna e o muco secretado por estas evitam a perda de água (Melo & Amaral, 2005).

Figura 1: Anêmona-do-mar *Bunodosoma cangicum.*
Fonte: http://uruguay1.blogspot.com

As anêmonas são pouco utilizadas como modelo biológico para contaminação, onde, na maioria das vezes, os estudos visaram analisar a utilização e propriedade das toxinas presentes nos nematocistos, a relação simbiótica desses animais com as zooxantelas, em alguns casos utilizando contaminantes a fim de observar se há diminuição da simbiose ou bioacumulação (Harland & Nganro, 1990; Cline *et al.*, 1995; Martínez *et al.*, 2002; Greenwood *et al.*, 2003; Mitchelmore *et al.*, 2003a; Mitchelmore *et al.*, 2003b, Homna *et al.*, 2003; Kuo *et al.*, 2010; Wolenski *et al.*, 2013). Sob os aspectos da fisiologia destes organismos, o conhecimento é bastante restrito. Mendes (1976) caracterizou quimicamente a junção neuromuscular; outros estudos dizem respeito à toxina liberada pelo animal, desde aspectos químicos até mecanismos de ação (Malpezzi *et al.*, 1993; Ale *et al.*, 2000; Oliveira *et al.*, 2004). Outra linha de estudo visa os processos de regulação osmótica e iônica destes animais, como regulação do volume celular por meio de osmólitos

orgânicos e inorgânicos, já que estes vivem em regiões susceptíveis a grandes variações de salinidade (Male & Storey, 1983; Deaton & Hoffmann, 1988; Amado *et al.*, 2011). No entanto, é importante conhecer o modo que estes organismos reagem frente à exposição por poluentes, visto que são animais de pouca mobilidade que não possuem a capacidade de fuga das adversidades, ficando expostos à poluição.

O cobre (Cu) é um micronutriente essencial requerido por todos os organismos vivos, utilizado em diversos processos fisiológicos e bioquímicos, entretanto, é considerado potencialmente tóxico aos organismos aquáticos quando em excesso na água (Moore, 1985; Engel and Brouwer,1987; Rainer and Brouwer, 1993; Martins *et al.*, 2011). Ele é um metal comumente encontrado em ecossistemas marinhos, chegando até estes por escoamento de áreas de mineração, descargas de esgotos, tratamentos de efluentes industriais, tintas anti-incrustantes usadas em embarcações, refinarias, etc (Bryan and Hummerstone, 1971; Guzmán and Jiménez, 1992; Jones, 1997; Mitchelmore *et al.*, 2003a; Niencheski *et al.*, 2006; D'Adamo *et al.*, 2008; Main *et al.*, 2010).

Este metal por ser comum ambientes marinhos, é regulamentado pelo Conselho Nacional do Meio Ambiente (CONAMA), através da resolução nº 357/05, a qual estabelece os limites máximos permitidos para o lançamento de cobre em águas salinas. Determina que para águas de classe I (destinadas à recreação de contato primário, à proteção das comunidades aquáticas e à aquicultura e a atividade de pesca) o limite de cobre dissolvido na água é 5µg.L^{-1} e para classe II (destinadas à pesca amadora e a recreação de contato secundário) é 7,8 µg.L^{-1}.

O limite é dado como cobre dissolvido, pois este é um metal de transição que pode passar da forma oxidada cúprica (Cu^{2+}) para a reduzida cuprosa (Cu^{+}), podendo então aceitar ou doar elétrons. Devido a esta atividade redox, o cobre é um cofator essencial em muitas vias enzimáticas, incluindo a oxidação respiratória, síntese de neurotransmissores, o metabolismo do ferro, e pigmentação. Esta mesma atividade redox pode catalisar a reação de Fenton, produzindo espécies reativas de oxigênio (ERO) (van den Berghe & Klomp, 2010).

Nas reações de Haber-Weiss e Fenton (Fig. 2), a oxidação do cobre conduz a conversão do peróxido de hidrogênio ao ânion hidróxido e ao radical hidroxila. Este metal também pode reagir com o ânion superóxido para produzir radicais de oxigênio. Quando há aumento dos estresses abióticos no ambiente, os mecanismos de transportes de elétrons tornam-se menos eficientes. Por sua vez, elétrons livres são mais prováveis de interagir como oxigênio diatômico molecular, resultando em uma série de espécies reativas de oxigênio (Main *et al.*, 2010).

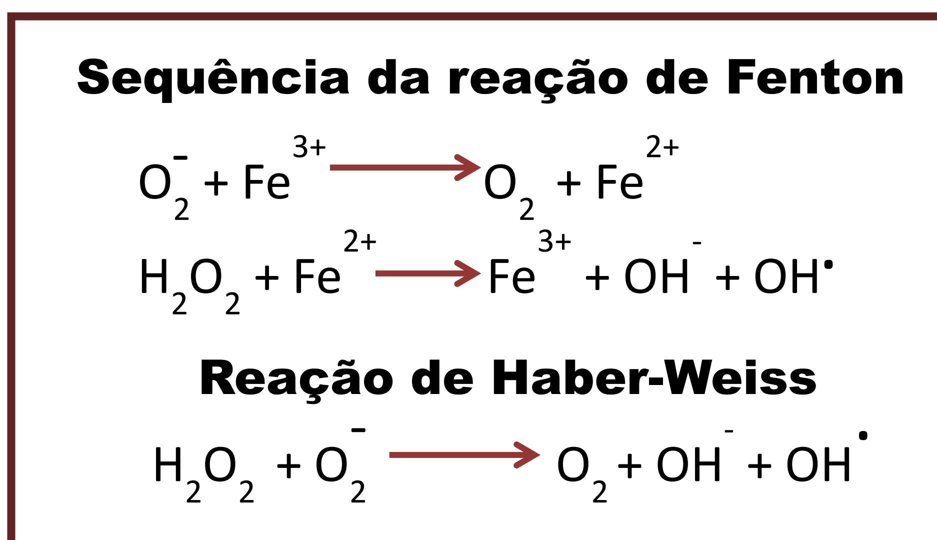

Figura 2: Formação de espécies reativas de oxigênio a partir das reações de Fenton e Haber-Weiss.
Fonte: adaptado de http://imaging.onlinejacc.org

Em diferentres organismos aquáticos existem evidências de que a toxicidade do cobre através do aumento de espécies reativas de oxigênio causa estresse oxidativo, evidenciado pela presença de dano ao DNA, alteração de defesas antioxidantes, além de efeitos secundários (Lee *et al.*, 2010). Por exemplo, Liu e Colaboradores (2005), ao exporem o peixe *Carassius auratus* ao cobre por 40 dias, viram um aumento significativo nas defesas antioxidantes superóxido dismutase e glutationa-s-transferase (SOD e GST). Existe o conceito que o íon cobre, na presença de agentes redutores, causa diversos tipos de danos ao DNA e também estão aptos a ajudar outros agentes causadores de dano, através da produção de ERO. Os tipos de danos incluem: oxidação das bases púricas e pirimídicas, mais notadamente da guanina formando 8-oxoGuanina, formação de ligações cruzadas entre bases

adjacentes, resultando em mutações duplas; além de quebras duplas e simples na fita de DNA (Linder, 2012).

O metabolismo celular naturalmente gera ERO, que são neutralizadas através de antioxidantes. No processo de formação de energia, a cadeia transportadora de elétrons gera espécies reativas de oxigênio. Evolutivamente, foram selecionadas várias estratégias antioxidantes para as células lidarem com a toxicidade do oxigênio. Os agentes considerados como antioxidantes compreendem enzimas que removem cataliticamente radicais e espécies reativas (por exemplo as enzimas superóxido dismutase, glutationa redutase, glutationa peroxidase e catalase); proteínas que minimizam a disponibilidade de pró-oxidantes (por exemplo transferrinas, metalotioneínas, ferritinas); proteínas que protegem processos celulares contra danos oxidativos através de outros mecanismos não enzimáticos, como as proteínas de estresse; moléculas de baixo peso molecular que possuem a capacidade de captar ERO via auto-oxidação, como o tripeptídeo glutationa e aquelas que possuem grupo tiol (SH), ou vitaminas como α-tocoferol, ácido ascórbico e β-caroteno (Barreiros *et al.*, 2006; Berra *et al*, 2006).

Quando ocorre um desbalanço entre o número de espécies reativas de oxigênio formadas e a neutralização destas, com a ocorrência de dano, diz-se que a célula está sob estresse oxidativo (Bandyopadhyay *et al.*, 1999; Berra *et al.*, 2006; Paital & Chainy, 2012, Lesser, 2012).

Em sistemas biológicos, a membrana celular, a qual tem função vital para a célula pois mantém a homeostasia dos organismos, constitui um dos alvos de atuação de ERO, promovendo modificações físicas nestas membranas que resultam em alterações na fluidez e permeabilidade, comprometendo suas funções biológicas. Além da membrana que envolve a célula, as membranas das organelas intracelulares, tais como mitocôndria, retículo endoplasmático, núcleo etc., apresentam uma estrutura bilipídica e uma variedade de proteínas e açúcares. O dano celular resulta basicamente de ataque de ERO sobre as macromoléculas, tais como açúcares, DNA, proteínas e lipídios (Berra *et al.*, 2006; Vasconcellos *et al.*, 2007; Sandrini *et al.*, 2009).

Em hepatócitos de peixes, Sandrini e Colaboradores (2009) encontraram maiores concentrações de ERO e maior expressão de genes ligados ao reparo de DNA em peixes *Danio rerio* exposto ao cobre; já com invertebrados,

Gabbianelli e colaboradores (2003) visualizaram uma indução de dano à molécula de DNA pelo cobre no molusco bivalve *Scapharca inaequivalvis.* Quanto aos cnidários, Schwarz e colaboradores (2013) ao exporem corais a diferentes concentrações de cobre viram que esse metal leva os animais a uma resposta de estresse oxidativo com subsequente dano ao DNA.

Quando um organismo está exposto a uma substância no ambiente, há a possibilidade de acumulação desta no seu corpo. Essa capacidade de acumular uma substância, através do meio circundante ou de seu alimento por um dado organismo, chama-se bioacumulação (Esser, 1986). Essa bioacumulação pode levar o animal a uma maior susceptibilidade ao estresse oxidativo (Harland & Nganro, 1990; Main *et al.*, 2010), gerando uma necessidade de eliminar o excesso da substância do organismo.

A fim de prevenir a célula de danos provocados por substâncias exógenas, conhecidos como xenobióticos, existe uma superfamília de proteínas transmembranas ABC (ATP-*binding cassete*), as quais realizam o transporte de substâncias para fora das células com gasto de ATP. Os transportadores ABC são altamente conservados entre os vertebrados e invertebrados (Bard, 2000; Buss & Callaghan, 2008).

Dentro desta superfamília de proteínas (ABC) encontra-se a glicoproteína P (Pgp), um potencial biomarcador de defesa celular, que gera o fenótipo de resistência a múltiplos xenobióticos (MXR) (Kurelec *et al.*, 2000; Sandrine & Marc, 2007). A indução do fenótipo MXR pode aumentar a resistência individual ou de populações em águas poluídas. Portanto, avaliar o nível de indução MXR em indivíduos/populações pode ser usado como um biomarcador de susceptibilidade para estes organismos (Kurelec *et al.*, 2000).

Em invertebrados, como esponjas, vermes e moluscos, este mecanismo já foi estudado (Bard, 2000; Eufemia & Epel, 2000; Pessati *et al.*, 2002; Achard *et al.*, 2004; Zaja *et al.*, 2006; Rocha & Souza, 2012). Mariscos expostos a cádmio por 15 dias, por exemplo, apresentam uma indução de MXR, atingindo o máximo de atividade no 8º dia. Respostas semelhantes foram encontradas para exposição a outros metais, como mercúrio, zinco e cobre, onde Hg foi responsável pela maior indução de MXR; e o Cu apresentou na menor bioacumulação tecidual o maior poder de indução da proteína (Achard *et al.*, 2004). Alguns xenobióticos, como óleo diesel, podem inibir esse mecanismo de

defesa. Tais inibidores de MXR, por exemplo, podem aumentar a acumulação de substâncias cancerígenas em mexilhão, com realce subsequente da produção de seus metabólitos mutagênicos, na indução de quebras de fita simples no DNA e na indução de adutos de DNA (Smital & Kurelec, 1998). Em cnidários, este mecanismo já foi identificado em corais. Venn e Colaboradores (2009) viram uma maior expressão de proteína Pgp naqueles organismos que foram expostos a contaminantes ambientais, inclusive ao cobre.

Esse tipo de mecanismo é importante para auxiliar os organismos na remoção dos xenobióticos. Uma dificuldade no mecanismo de extrusão de contaminantes pode levar a um estresse oxidativo, podendo gerar então um dano genético, muitas vezes irreversível.

A indução de danos oxidativos nas bases do DNA ocorre a partir da sua reação com ERO. Essas lesões podem ocorrer devido à oxidação direta dos ácidos nucléicos ou, muitas vezes, podem levar à formação de quebras em uma das cadeias do DNA (quebras simples - SSB "single strand break") ou quebras simples em posições aproximadamente simétricas nas duas cadeias do DNA (quebras duplas - DSB "double strand break"). Além disso, quebras simples podem gerar quebras duplas durante a replicação celular (Berra *et al.*, 2006). Uma maneira de visualizar estas quebras é através do ensaio cometa, uma técnica bastante conveniente e sensitiva para células individuais. Os programas de análise através de imagens, permitem a quantificação de vários parâmetros de cometa, comumente mensurando o tamanho da cauda e o momento da mesma. Essas duas medidas são mais propensas a diferenças apresentadas no gel de agarose, portanto, muitos autores usam o percentual de DNA na região da cauda como critério para quantificação das quebras na molécula (Mitchelmore & Chipman, 1998).

Na literatura dificilmente se encontra trabalhos utilizando metais em invertebrados, evidenciando estresse oxidativo, expressão de MXR e dano de DNA ao mesmo tempo. Apenas estudos envolvendo alguns desses aspectos foram encontrados, como por exemplo, cobre, estresse oxidativo e dano de DNA (Scharwz *et al.*, 2013); cobre e MXR em corais (Venn *et al.*, 2009), chumbo e MXR em bivalves (Rocha & Souza, 2012).

A fim de diminuir o impacto ambiental, que a geração de resíduos decorrentes de experimentos ecotoxicológicos *in vivo* podem trazer, técnicas

utilizando experimentos *in vitro* vem sendo desenvolvidas. A ideia vem da premissa que a citotoxicidade de uma substância causa efeitos adversos na estrutura e/ou funcionalidade de um ou mais constituintes celulares, dessa maneira, estudos utilizando a própria célula prediriam posteriores efeitos a níveis mais elevados, como no próprio organismo, sem haver necessidade da utilização do indivíduo (Garle *et al.*, 1994, Evans *et al.*, 2001; Kienzler *et al.*, 2012).

Tendo em vista que as anêmonas são animais bentônicos de pouca mobilidade, por isso, são candidatos mais susceptíveis à exposição aos contaminantes presentes na água, é importante conhecer mais sobre a maneira como reagem à poluição aquática, objetivando obter mais dados sobre sua fisiologia frente a mudanças no ambiente. Além disso, não existem dados sobre cultura de células de *Bunodosoma cangicum* na literatura.

Objetivo geral:

Estudar a capacidade de defesa e possível dano ao DNA à toxicidade causada pelo cobre em células de anêmonas *Bunodosoma cangicum*.

Objetivos específicos:

- Estabelecer uma cultura primária de células de anêmonas;
- Avaliar a citotoxicidade do cobre
- Avaliar a indução do fenótipo MXR;
- Avaliar a quantidade de espécies reativas de oxigênio formadas;
- Avaliar dano ao DNA, através de ensaio cometa.

Cell damage and cell defense induced by copper: an explant model to study anemone cells

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Running Head: cell defense and damage

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*Este manuscrito será submetido ao periódico **Toxicology*** **In vitro**
O manuscrito está apresentado aqui no formato editado

Abstract

Sea anemones are benthic organisms, of low mobility and can be directly affected by water pollution. This work studied the defense mechanisms and DNA damage caused by copper toxicity in cells from the anemone *Bunodosoma cangicum*. For this, exposure of anemones cells were held, kept in primary culture through explant of podal disk to copper (7.8 and 15.6 µg L^{-1}), and the control group, for 6 and 24h. Cytotoxicity was seen through the viability and cell number, MXR phenotype through the accumulation of Rhodamine-B, ROS generation by H_2DCF-DA and DNA damage by comet assay. The results obtained show that there is a drop in viability and number of cells, especially after exposure of 24h in 15.6 µg L^{-1}. There is an induction of the MXR activity only at 7.8 µg L^{-1} for 24h. As for ROS, there is an increase in the generation of reactive species in greatest concentration of copper for 6h, and in both for 24h, which leads to oxidative stress, which culminates with a DNA damage. What was evidenced by the increase of the tail size, % DNA presented and moment of tail. Therefore, the copper represents an adversity to the anemones cells, being cytotoxic and genotoxic.

1. Introduction

Sea anemones are solitary organisms, that feed on small prey present in the plankton or absorb dissolved organic matter and often possess interactions with symbiotic photosynthetic organisms (de Capitani, 2007). They are benthic osmoconformers, of low mobility, occupying intertidal regions, shallow coastal reefs, and rocky shores of continental regions to Oceanic Islands and atolls (Melo & Amaral, 2005).

Bunodosoma cangicum is a sea anemone commonly found in the intertidal area of the Southeast regions of Brazil. They tend to fasten onto the rocks or in sandy crevices, where they remain buried in the sand exposing only their oral disk (Melo & Amaral, 2005).

Anemones are little used as biological models for contamination, and for the most part, studies of anemones seek to examine the use and properties of

the toxins present in the nematocysts or describe the symbiotic relationship of these animals with zooxanthellae (Cline *et al.*, 1995; Greenwood *et al.*, 2003; Harland & Nganro, 1990; Homna *et al.*, 2003; Kuo *et al.*, 2010; Martínez *et al.*, 2002; Mitchelmore *et al.*, 2003a; Mitchelmore *et al.*, 2003b; Wolenski *et al.*, 2013). Considering the physiology of these organisms, knowledge is quite restricted, work has been focused on the characterization of the neuromuscular junction; function and activation of nematocysts; and osmoregulatory processes involved in cell volume regulation, through organic and inorganic osmolytes (Amado *et al.*, 2011; Male & Storey, 1983; Malpezzi *et al.*, 1993; Mendes, 1976; Oliveira *et al.* 2004). However, it is important to understand how these organisms react to exposure by pollutants, since they are animals of little mobility that do not have the ability to escape adverse conditions. Copper (Cu) is an essential nutrient required by all living organisms, important in several physiological and biochemical processes, however, it is considered potentially toxic to aquatic organisms when in excess in the water (Engel and Brouwer, 1987; Martins *et al.*, 2011; Moore, 1985; Rainer and Brouwer, 1993). It is a metal commonly found in marine ecosystems, originating from mining areas, sewage discharges, industrial effluent treatments, anti-fouling paints used on ships, refineries, etc (Bryan and Hummerstone, 1971; D'Adamo et al., 2008; Guzmán and Jiménez, 1992; Jones, 1997; Main *et al.*, 2010; Mitchelmore *et al.*, 2003a; Niencheski et al., 2006).

This is a transition metal that can go from the oxidized copper form (Cu^{2+}) to reduced cuprous (Cu^{+}), and may accept or donate electrons. Due to this redox activity, copper is an essential cofactor for many enzymatic pathways, including respiratory oxidation, neurotransmitter synthesis, the metabolism of iron and pigmentation. This same redox activity can catalyze the Fenton reaction, producing reactive oxygen species (ROS) (van den Berghe & Klomp, 2010).

When there is an imbalance between the number of reactive oxygen species formed and the neutralization of these by antioxidant defenses, changes in cellular signals and some type of damage, it is said that the cell is under oxidative stress (Bandyopadhyay *et al.*, 1999; Berra *et al.*, 2006; Lesser, 2012; Paital & Chainy, 2012). In marine organisms there is evidence that the

toxicity of copper causes oxidative stress, an increase in antioxidants and DNA damage (Lee *et al.*, 2010).

In biological systems, the cell membrane, is one of the *foci* of ROS activity. More than the membrane that surrounds the cell, intracellular organelles membrane, such as mitochondria, endoplasmic reticulum, nucleus etc., they present a bilipid structure and a variety of proteins and sugars. Cellular damage results basically from ROS attack on macromolecules such as sugars, DNA, proteins and lipids (Berra *et al.*, 2006; Sandrini *et al.*, 2009; Vasconcellos *et al.*, 2007).

Generally, when an organism is exposed to a particular substance, it tends to accumulate in their tissues. This bioaccumulation can lead to an increase of reactive oxygen species, and in order to prevent oxidative stress, organisms need to export such substances (Esser, 1986; Harland & Nganro, 1990; Main *et al.* 2010).

In order to prevent cell damage caused by exogenous substances, known as xenobiotics, there is a superfamily of transmembrane proteins, denominated ABC (ATP-binding cassette), ABC transporters are highly conserved among vertebrates and invertebrates (Bard, 2000; Buss & Callaghan, 2008). Within this superfamily of proteins is the P-glycoprotein (Pgp), a potential biomarker of cell defense, which generates the resistance phenotype against multiple xenobiotics (MXR) (Kurelec *et al.*, 2000; Sandrine & Marc, 2007). The induction of MXR can increase individual or populational resistance in polluted waters. Therefore, assessments of the level of MXR induction in individuals/populations can be used as a biomarker of susceptibility in these organisms (Kurelec *et al.*, 2000).

An inhibition in the extrusion mechanism of contaminants may lead to oxidative stress, and can then generate genetic damage, which is often irreversible. In the relevant literature, one may find works involving copper, oxidative stress and DNA damage (Schwarz *et al.*, 2013); copper and MXR in coral (Venn *et al.*, 2009), lead and MXR in bivalves (Rocha & Souza, 2012); however, finding works using metals in invertebrates, showing oxidative stress, and MXR expression linked to DNA damage are not common.

Studies using contaminants for identifying the toxic effects that these substances may cause, can generate a considerable amount of waste. Which

may then be discarded into the environment. In order to reduce the environmental impact that in vivo toxicological experiments may bring, techniques using *in vitro* experiments have been developed. As cytotoxicity can cause damage to the structure and/or functionality of one or more cellular compounds, studies using isolated cells may predict effects at the tissue organ and organismal level, without the need of using the individual (Evans *et al.*, 2001; Garle *et al.*, 1994; Kienzler *et al.*, 2012).

Since anemones are benthic animals of little mobility, it is important to know more about the way they react to water pollution, aiming to obtain more data about their physiological responses to changes in the environment. In addition, there are no data in the literature on cell culture of anemones as toxicological models. Therefore, the objective of this work is to study the defense capability and possible damage to DNA resulting from copper toxicity in isolated cells of anemones *Bunodosoma cangicum*.

2. Materials and methods

2.1. Collection and maintenance of anemones

The sea anemones, *Bunodosoma cangicum* Corrêa 1964, were collected in the intertidal area (32° 09 ' 40.25 '' S; 52° 05 ' 51.96 '') of Cassino Beach (Rio Grande, RS, Brazil). The animals were collected manually and were packed in boxes with seawater, and transported to the laboratory.

The animals were kept in glass aquariums, with ≈ 20ºC temperature, constant aeration, and photoperiod 12L: 12D, salinity 30 ‰. The animals were fed each week with pieces of fish on the oral disk.

2.2*. Primary culture of cells from* Bunodosoma cangicum

After preliminary testing, explants of pedal disk tissue fragments were determined to be the best method for the primary culture of cells.

Anemones were placed in saline solution, compatible with the animal fluid at 30 ‰ (in mM: 399.7 NaCl, 8.8 KCl, 8.8 $CaCl_2$, 45.9 $MgSO_4$, 2.2

$NaHCO_3$, pH 7.4, ≈ 800 mOsm. Kg H_2O^{-1}) added supplemented with 200 mM magnesium chloride as an anaesthetized. After 30 min, pieces of tissue next to the pedal region were removed. After removal of the tissue, the anemones were returned to the aquarium to recover.

The tissue was placed in PBS (Phosphate Buffer Saline) solution (in mM: 340 NaCl, 12 Na_2HPO_4, 2.2, KH_2PO_4, 16 KCl and 5 EDTA – calcium free, pH 7.4, ≈ 800 mOsm. Kg H_2O^{-1}), added with H_2O_2 (30 µl for each 5 mL of PBS) and taken to the laminar flow. The material was then cut into small fragments (~ 3 mm^3) in a petri dish containing PBS with hydrogen peroxide and placed in a beaker containing PBS without hydrogen peroxide and taken to an agitator, with rotational movements of 80 rpm, for 20 minutes to loosen tissue cells more easily. After this time, tissue fragments were selected and placed in microplates of 24 wells (for culture), covered by 1 mL of culture medium M199 (Sigma-Aldrich, USA - enriched with $NaHCO_3$ 41 mM, KCl 80 mM and NaCl 205 mM) for adjustment of osmolality (≈ 800 mOsm. Kg H_2O^{-1}, in a way equivalently to the celenteron fluid) and 3% antibiotic/antimitotic.

These plates were then incubated at ± 20°C temperature. And the samples were observed over 10 days, to evaluate the number of cells and their viability.

2.3. *Exposure of cells to copper*

The cells were exposed to the metal on the third day of culture, this was based on initial observations of cellular viability and proliferation during the explant procedures. The cells were exposed for 6 and 24 h, in two concentrations of copper (added to the saline solution in the form of copper chloride) 7.8 $\mu g.L^{-1}$ Cu and 15.6 $\mu g.L^{-1}$ Cu (values referring to the CONAMA resolution N°. 357/2005, brazilian legislation, to saline water type II, and its double, respectively), and the saline solution (control – copper free). Before exposure and just after exposure, cell viability was evaluated through the exclusion test with trypan blue (0.08%), for analysis of cytotoxicity of metal. The experiments were conducted in 6 samples for each experimental condition.

2.4. MXR phenotype induction analysis

The test of rhodamine-B accumulation was performed as described by Kurelec and Collaborators (2000), modified for cell culture. Rhodamine is an ABC transporter substrate, so fluorescent accumulation was used as indicative of the activity of these proteins. The cells had the culture medium removed and were incubated in rhodamine-B (RB, Sigma-Aldrich, USA) 10 µM in saline solution, using 2- $3x10^5$ céls.mL^{-1} cells for each sample. They were incubated for one hour at room temperature. After exposure the cells were washed 2 times in saline solution to remove the excess RB, then were placed in opaque microplates (250 µl per sample) and the fluorescence was analyzed, with excitation and emission of 485 and 590 nm, respectively (Victor 2, Perkin Elmer). The results were expressed in concentration of rhodamine calculated from a standard curve of fluorescence of rhodamine, and normalized by the number of cells from each sample. For the standard curve were used concentrations of 1; 0.1; 0.05; 0.02 and 0.01 µM.

2.5. Reactive Oxygen Species Analysis

The protocol followed was proposed by Viarengo and Collaborators (1999), with modifications as those found in Sandrini and Collaborators (2009). The samples, each containing $3x10^5$ cells.mL^{-1}, after incubation with the metal treatments were washed in PBS (as above) and then incubated at room temperature in PBS + 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich, USA) for 30 minutes. After this time the cells were washed again and transferred as 160 µl samples to 96 well microplates. The ROS were detected by fluorescence, excitation of 485 nm and emission of 520 nm (Victor 2, Perkin Elmer), with a period of reading of 2 h, carried out every 5 min. The results are presented as integrated area under the fluorescence curve, and normalized by cell number.

2.6. Comet assay

This test was performed according to the protocol described by Mitchelmore and Collaborators (1998), with some modifications that were performed by Mitchelmore & Hyatt (2004), which adapted the method to anemones.

After experimental exposure, the cells were washed in saline solution for removal of copper. In this assay, 80 µl of cell suspension ($4\text{-}7x10^5$ $cells.mL^{-1}$) was dissolved in 60 µl of low melting point agarose 1% and settled on a slide previously coated with normal melting point agarose 1.5%. This sample was covered with a coverslip and allowed to cool for 10 min. After, the coverslip was removed and the slides submerged in a solution of cell lysis buffer (2.5 $mol.L^{-1}$ NaCl, 100 $mmol.L^{-1}$ EDTA, 10 $mmol.L^{-1}$ Tris, 1% Triton X-100, 10% DMSO, pH 10.0) at 4ºC. After the lysis, the slides were washed 3 times (5 minutes each) in saline solution and then placed in a horizontal electrophoresis chamber to incubate in the denaturing buffer (1.0 $mmol.L^{-1}$ EDTA, 300 $mmol.L^{-1}$ NaOH, pH 13.0) for 25 minutes. This buffer was at maintained at 4ºC, placed on a tray with ice, and protected from light. Electrophoresis was performed for 25 min, at 1 $V.cm^{-1}$, 300 mA. After which , the slides were neutralized, by rinsing 3 times (5 minutes each) in (0.4 mM Tris, pH 7.5) and then fixed using absolute ethanol. To mark the genetic material we used the dye Sybr ® Safe DNA Gel Stain. The slide were imaged on a fluorescence microscope (Olympus BX50), 400 x magnification, further analysis, was performed by the open source program (Image J), using the plugin (macro) Comet Assay. For each treatment (control, 7.8 $µg.L^{-1}$ Cu and 15.6 $µg.L^{-1}$ Cu) 100 nuclei were used, analyzing the tail size, percentage of DNA on the tail and the tail moment (size x percent).

2.7. Statistical analysis

From the results of the different evaluated parameters were calculated mean and standard error (SE) followed by analysis of variance (ANOVA) and Tukey post hoc test, where were considered significantly different values of p ≤

0.05. When necessary a *T* test was performed, considering significant values of $p \leq 0.05$.

3. Results

For the establishment of primary cell culture of anemones, through the explant of fragments of pedal disk, preliminary experiments were performed for monitoring the cell number and viability. The cultures were maintained and analyzed over 10 days, generating the results below (the first day correspond to culture after 24 h).

As noted in Figure 1, the number of cells does not vary significantly at any time point, where number of cells remains around $2x10^5$ cells.mL^{-1} since Day 1 (first day culture) until the 10th day of culture ($p > 0.05$, n= 5-8). With regard to viability, during the 10 days of maintenance it remained in the range of 90%, without significant variation throughout the period ($p > 0.05$; figure 2).

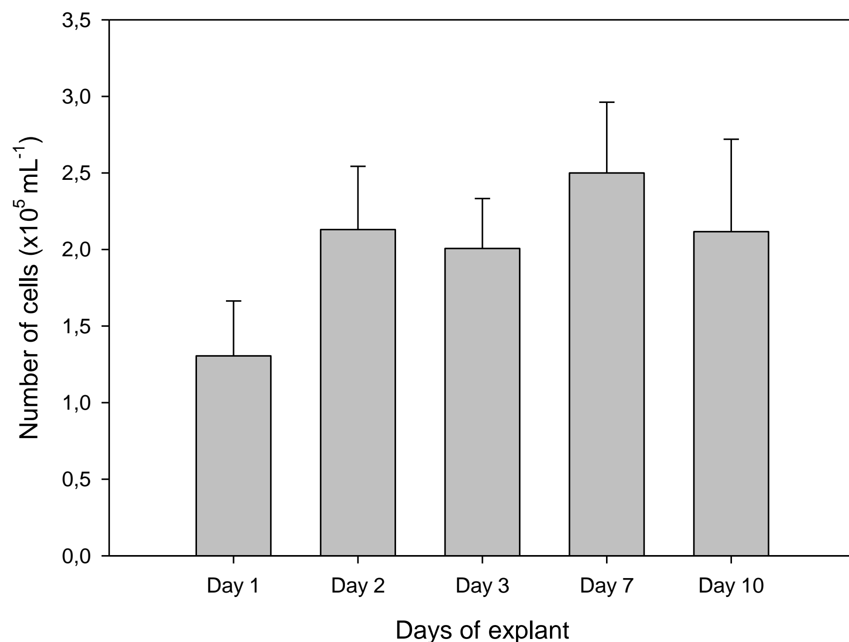

Figure 1. Average number of cells of *B. cangicum* in primary culture from explant pedal disk (mean ± se).

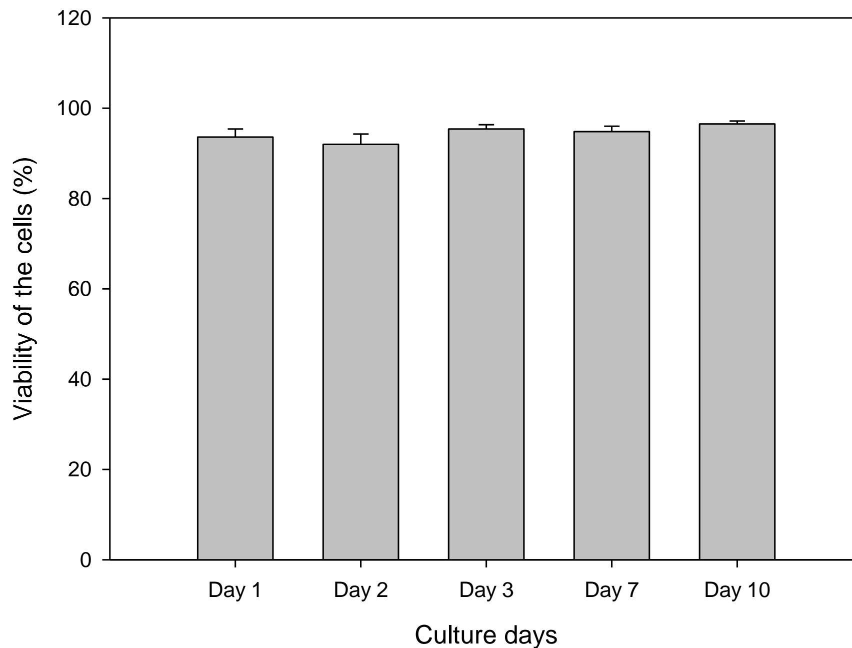

Figure 2. Viability (%) of cells of *B. cangicum* in primary culture from explants pedal disk (mean ± se).

With the data obtained from cell number, it was established that experiments with copper exposure would be performed at 3 days of culture.

Analysis of cytotoxicity

The cytotoxicity of copper was analyzed by means of cell viability, according to cell membrane integrity, which exhibited the results shown in Figure 3. During the experimental time of 6 h, we observed a 15% drop in viability when cells were exposed to copper 15.6 $\mu g.L^{-1}$ ($p < 0.05$, $n = 6$). Considering cells exposed for 24 h, at a copper concentration of 7.8 $\mu g.L^{-1}$, there was a 27% drop in viability; but at the copper concentration of 15.6 $\mu g.L^{-1}$, it was possible to observe a 40% decrease in viability ($p < 0.05$, $n = 6$).

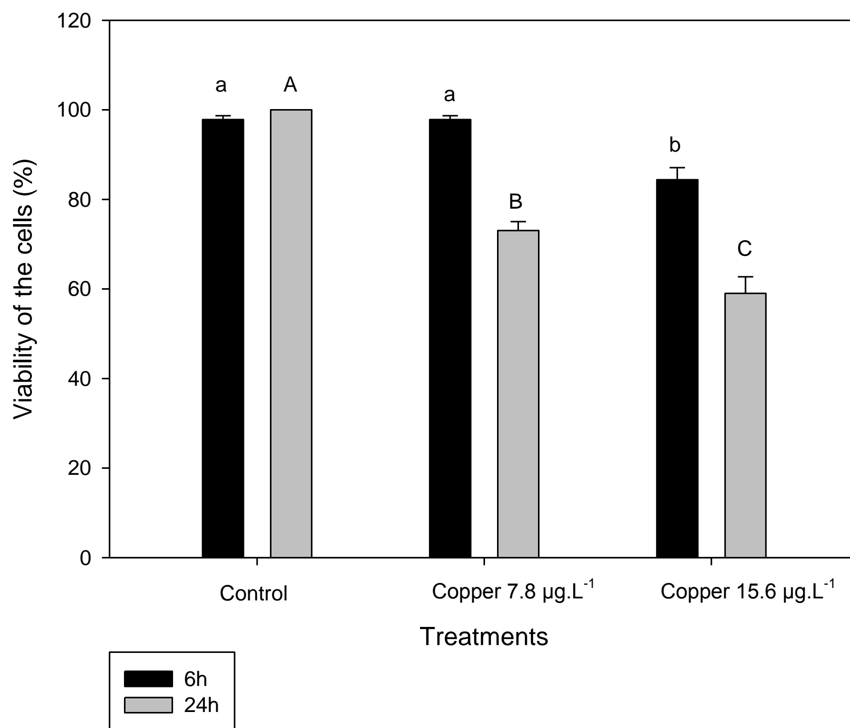

Figure 3. Viability of cells of *B. cangicum* exposed to copper. Lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the exposure for 24 h.

The cytotoxicity of copper was also observed through total cell number. As can be seen in Figure 4, the number of cells remained constant in those exposed for 6 hours, both in copper concentrations of 7.8 $\mu g.L^{-1}$ as well as 15.6 $\mu g.L^{-1}$, however, after 24 h exposure there was a significant reduction of 36% (< 0.05, n = 10-13) in those exposed to copper 15.6 $\mu g.L^{-1}$.

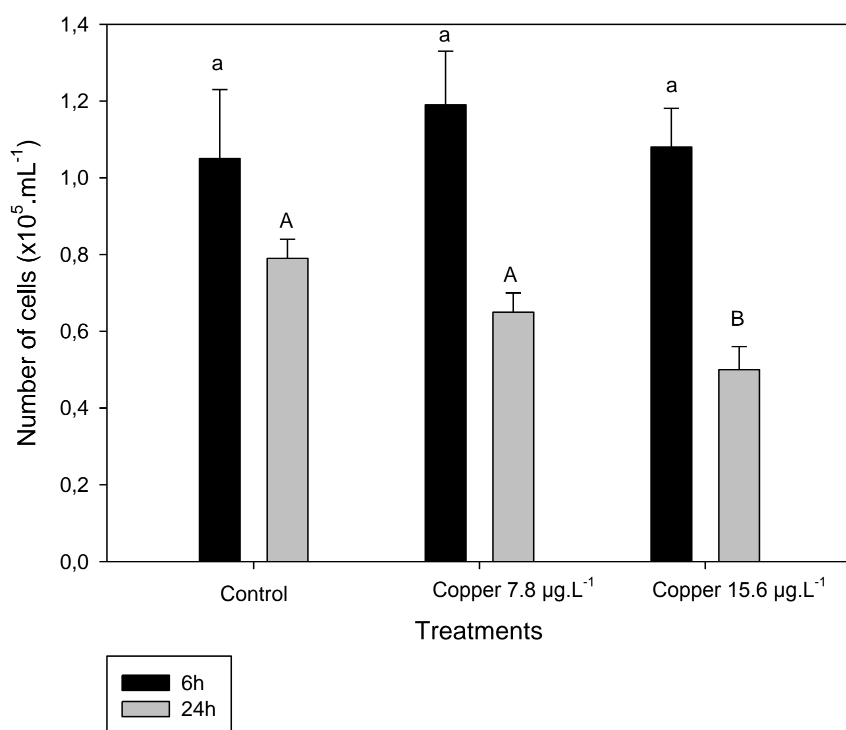

Figure 4. Number of cells of *B. cangicum* exposed to copper. Different lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the cells after exposure for 24 h to copper.

MXR phenotype induction analysis

In relation to the phenotype of multiple xenobiotic resistance (MXR), the results obtained are shown in Figure 5. Based on the standard curve used it was possible to observe that when the anemones cells were incubated with 10 µM of rhodamine B, for 30 minutes, the final concentrations were $6.1623^{-7} \pm 1.62^{-7}$ and $4.1812^{-7} \pm 0.826^{-7}$ µM.cells^{-1} (mean ± standard deviation), for 6 and 24 hours of exposure, respectively. At 6 h of copper exposure it was not seen any significant difference between treatments in relation to the control group ($p < 0.05$, $n = 5$). When exposed for 24 h, it was possible to observe an activation of the resistance mechanism (by about 53%) compared to the control group in those cells exposed to 7.8 µg.L^{-1} at the same time experimental (4.1812^{-7} e 1.9638^{-7} µM.cells^{-1}, for control and 7.8 µg.L^{-1}, respectively; $p < 0.05$). At the concentration of 15.6 µg.L^{-1} there was no significant difference between the control group and the lower copper concentration.

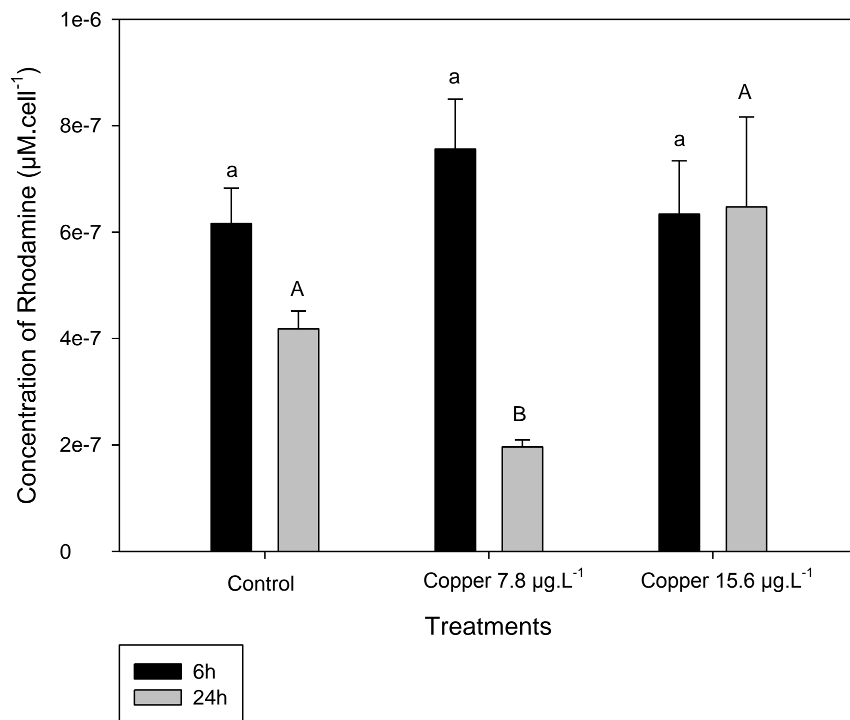

Figure 5. RB concentration by *B. cangicum* cells exposed to copper. Different lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the exposure for 24 h.

Analysis of ROS

In the analysis of the Reactive Oxygen Species generation by exposure to copper (Figure 6), we observed a significant increase of 11.5% in the generation of ROS in cells exposed for 6 h in the highest concentration of copper 15.6 $\mu g.L^{-1}$ ($p < 0.05$). In cells that were exposed for 24 h, the significant increase in the amount of reactive species can already be in concentration of 7.8 $\mu g.L^{-1}$, increasing to about 20% when exposed to 15.6 $\mu g.L^{-1}$ ($p < 0.05$, n = 4-5).

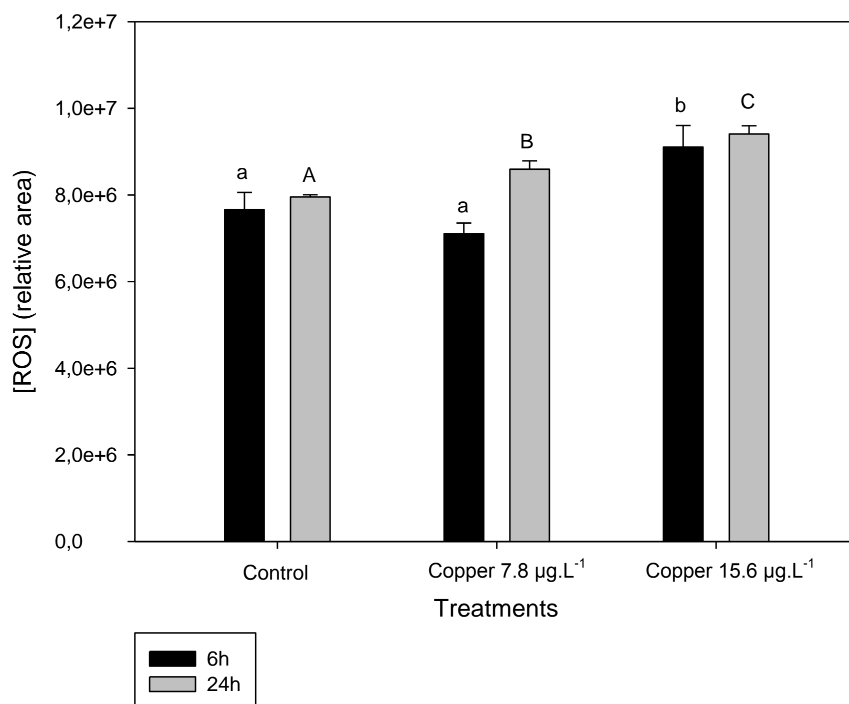

Figure 6. Amount of ROS (mean area ± se) in the cells of *B. cangicum* exposed to copper. Different lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the exposure for 24 h.

DNA damage analysis

The results generated by exposure to copper (analysis at the Image J software with "Comet Assay" macro) evaluate the damage to DNA on about 3 variables, tail size, percentage of DNA in this tail and tail moment, which is the multiplication of previous variables. Figures 7 and 8 were generated, showing the size of the tail and the percentage of DNA present, respectively. The tail moment is shown in table 1. The number of cells was not enough for analysis in experiments in which cells were exposed for 24 hours at a concentration of 15.6 $\mu g.L^{-1}$ Cu, so, the statistical analysis carried out on the experimental test of 24 hours was a student's T test (between control and 7.8 $\mu g.L^{-1}$).

As for the size of the tail, we observed that there was a significant increase only in those cells exposed to 15.6 $\mu g.L^{-1}$ Cu for 6 h ($p < 0.05$). Considering those cells exposed for 24 h, a significant difference was observed

in cells exposed to 7.8 μg.L^{-1} Cu compared to control group ($p < 0.05$), where the cells exposed to the metal showed a 50% increase in the size of the tail.

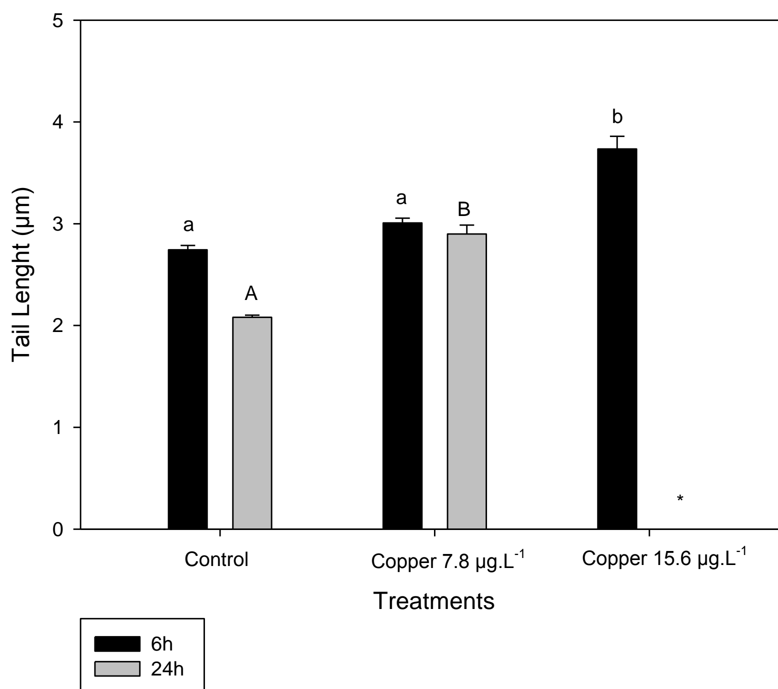

Figure 7. Average size of the tail of the cells of *B. cangicum* exposed to copper. Different lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the exposure for 24 h. * In the concentration 15.6 μg.L^{-1} it was not obtained sufficient number of cells.

In relation to the percentage of DNA present in the tail, it was possible to observe that for those cells exposed for 6 h, the percentage doubled and quadrupled at 7.8 and 15.6 μg.L^{-1} Cu, respectively ($p < 0.05$; $n = 3-4$). However, considering those that were exposed for 24 h, at a concentration of 7.8 μg.L^{-1} Cu, there was twice the percentage of DNA present in relation to the control group ($p < 0.05$; $n = 3-4$).

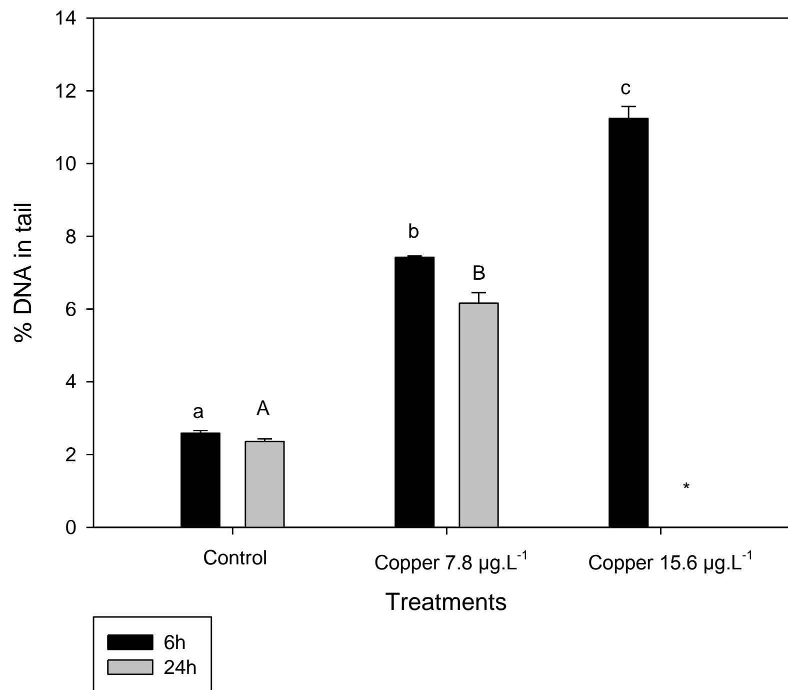

Figure 8. Percentage of DNA in the tail of cells of *B. cangicum* exposed to copper. Different lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the exposure for 24 h. * In the concentration 15.6 $\mu g.L^{-1}$ it was not obtained sufficient number of cells.

Regarding the tail moment, the observed variations are the same as those seen in relation to the percentage of DNA present in the tail. There was an increase of more than 100 and 200% for 7.8 and 15.6 $\mu g.L^{-1}$ Cu, respectively ($p < 0.05$; $n = 3\text{-}4$), in cells exposed for 6 h. Cells exposed for 24 h exhibited a tail moment 3 times higher in a concentration of 7.8 $\mu g.L^{-1}$ Cu than the control group ($p < 0.05$, $n = 3\text{-}4$).

Tail Moment (arbitrary units)		
Treatment	**6 h**	**24 h**
Control	7.79 ± 0.16 [a]	5.69 ± 0.27 [A]
7.8 $\mu g.L^{-1}$	20.66 ± 1.02 [b]	18.13 ± 0.09 [B]
15.6 $\mu g.L^{-1}$	45.67 ± 2.23 [c]	*

Table 1. Mean time of tail moment of *B. cangicum* cells exposed to copper. Different lowercase letters represent statistic difference ($p<0.05$) between treatments in the exposure for 6 h, and different capital letters indicate differences between treatments in the exposure for 24 h. * In the concentration 15.6 $\mu g.L^{-1}$ it was not obtained sufficient number of cells.

4. Discussion

For more than a decade, isolated nematocysts of sea-anemones have been used as a cellular model to study the mechanisms of cell volume regulation in anisosmotic situations (La Spada *et al.*, 1999; Marino and La Spada, 2007; Marino *et al.*, 2010). Employing cells from the podal disk of anemones, and obtained from the same cell dissociation technique, Amado and Collaborators (2011) conducted experiments of volume regulation in newly isolated cells, with a cell viability remaining around 90%. During the period from 1988 to 1998, primary cultures of marine invertebrates were performed basically in the phyla Porifera, Cnidaria, Crustacea, Mollusca, Echinodermata and Urochordata, however, even within these phyla only some orders were cultured, principally those of commercial interest (revision of Rinkevich, 1999). It is interesting to note that in Rinkevich's review, the author highlights the way in which the cultures were obtained, most commonly be cellular enzymatic dissociation protocols, although this method is probably more aggressive for cells as compare to our explant method and decreases cell viability, especially in cnidarians.

From about the year 2000 until today, the main primary cultures developed remain the same phyla, particularly molluscs and crustaceans (Cao *et al.*, 2004a; Cao *et al.*, 2004b; Cornet, 2006; Cornet, 2007; Domart-Coulon *et al.*, 2000; Jose *et al.*, 2011; Odintsova *et al.*, 2011). As for cnidarians, work with isolated cells from sea anemones have been developed, but only through

dissociation protocols (Amado *et al.*, 2011; Greenwood, 2003; Nagai *et al.*, 2002).Tissue explants are common in sponge cultures (Caralt *et al.*, 2003; Caralt *et al.*, 2007; Nickel & Brümmer, 2003), followed by molluscs and crustaceans (Jose *et al.*, 2011; Nambi *et al.*, 2012; Quinn *et al.*, 2009; Dirty & Dharmaraj, 2005; Dirty *et al.*, 2007;). However, this is the first reported use of anemones, this is the first reported in the literature. The technique developed in this work, of primary cell culture of anemones through pedal disk tissue explant, is a pioneer. It is observed that the results show a high viability, a high yield of isolated cells and a maintenance period of cell culture of more than a week, so this can be a simple, quick and safe methodology to be established as a model for *in vitro* analyses.

We have shown seen that copper presents cytotoxicity in anemones cells, showing a significant reduction in viability as well as cell numbers. Decrease in cell viability (measured through the Trypan blue exclusion method) was also found by Manzl and Collaborators (2004), when they exposed cells isolated from the gastropod *Helix pomatia* to copper and cadmium concentrations of 100 and 500 μM for 1 and 2 h. They assigned the toxicity of metals to an overload in the extrusion capacity of these contaminants by detoxifying enzymes, metallothionein in this case.

Our analysis of copper cytotoxicity was performed through Trypan blue exclusion, which evaluates the integrity of the cell membrane. As copper is a transition metal that increases the amount of reactive oxygen species and that these may affect the integrity of the cell membrane (Berra *et al.*, 2006; Vasconcellos *et al.*, 2007), this could provide a mechanism to explain this metal's cytotoxicity. The findings here with ROS support this hypothesis (results discussed below).

Shaligram and Campbell (2013) exposed 3 human cell lines to copper chloride and copper sulphate for 24 h and saw that at a concentration of 5 μM copper there was an increase in cell number and viability. However, when the copper concentration increased (50 and 500 μM) there was a reduction in both parameters. This increase in numbers and viability seen at the lower concentration, was explained by the fact that copper is an essential metal, and from the concentration of 50 μM and upward the metal began to become cytotoxic. However, by the results obtained here in this work, the concentration

of copper, which is much lower than those used in the works above (for example, 500 µM copper chloride is equivalent to 67 $\mu g.L^{-1}$) shows to be cytotoxic for anemones cells, confirming that this cell type is sensitive to copper.

In the multiple xenobiotics resistance phenotype, Venn and Collaborators (2009) saw an increase in gene expression of Pgp in corals that were exposed to different contaminants. Among the tested substances was copper, which generated an increase of almost 5 times in the protein expression of Pgp when exposed to 30 $\mu g.L^{-1}$ of the metal for 8 h. In isolated cells from gills of the bivalve *Corbicula fluminea* exposed to lead (1, 10 and 100 µM for 5 h), an increase in the amount of Pgp protein in these cells in concentrations of 10 and 100 µM, however, no significant difference was found in the MXR activity (Rocha & Souza, 2012). To evaluate the activity of Pgp protein, it is important to know the effective capacity of extrusion of the contaminants, however, if this appears unchanged, it does not necessarily mean that the mechanism as a whole is not being affected. The contaminant may not be inhibiting the activity, but might be changing the amount of protein allocated to the cell membrane, this result was observed in the cells of the bivalve. Unfortunately some contaminants behave as chemosensitizers of the MXR phenotype, compromising the defense ability (Kurelec *et al.*, 2000). Some substances, including metals, have the ability, in low concentrations, to stimulate defense systems and in higher concentrations to inhibit them. This biphasic response, of a dose-time characteristic, is known as homersis (Calabrese, 2008; Stebbing, 1981). This mechanism may explain the results found in this work as to the MXR phenotype in anemones cells exposed to copper, because in copper concentrations of 7.8 $\mu g.L^{-1}$ exposed for 24 hours, there was an increase in the activity of protein contaminant extrusion, and in the concentration of 15.6 $\mu g.L^{-1}$, although the difference has not been shown to be statistically significant, there is a tendency toward inhibition. In 6 hours exposure to metal we did not observe activation nor inhibition of this mechanism, which does not mean that the cells are not being stressed (reactive oxygen species and DNA damage increases). Achard and Collaborators (2004) found that by exposing the gills of the clam *Corbicula fluminea* to cadmium (15-60 $\mu g.L^{-1}$), they observed a higher induction of MXR as evidenced by less bioaccumulation. Copper also elicits homersis in hydrozoans, where low metal concentrations (10 $\mu g.L^{-1}$) stimulated the growth

of colonies of these animals (Stebbing, 2002), and the same pattern had already been seen by Stebbing (1981) at concentrations of 5 and 10 $\mu g.L^{-1}$ of copper. At higher concentrations (more of 10 $\mu g.L^{-1}$) the counteractive capacity is exceeded and inhibition of growth is observed. This type of behavior may be an adaptive response of the organism, which acts as a conditioning stimulus in order to protect against the damage of a life-threatening exposure later (preconditioning homersis) or to increase the protection/repair following a life-threatening exposure (post homersis) (Calabrese, 2008; Stebbing, 1981).

Considerable work has been undertaken to study metals and oxidative stress. Studies that measured antioxidant enzymes revealed that the metals, especially copper, cause an increase of these defenses, in particular affecting superoxide dismutase and catalase enzymes, suggesting that this metal causes oxidative stress in organisms leading to a defense response (Liu *et al.*, 2006, Main *et al.*, 2010; Semedo *et al.*, 2012). It is worth noting that in the work of Main and collaborators (2010) the animal studied was the *Aiptasia pallid* anemone, exposed to different concentrations of copper (0-50 $\mu g.L^{-1}$) for 7 days, their findings were that the activity of antioxidant enzymes increased from the concentration of 15 $\mu g.L^{-1}$ within 24 hours, suggesting that this metal causes an oxidative stress in these animals. These results corroborate with what we have observed in cells of *Bunodosoma cangicum*, where an increase in the copper concentrations and exposure time increases the amount of oxygen species produced, i.e. this metal, even within concentrations allowed by Brazilian law, interferes with their redox balance. Bopp and Collaborators (2008) used rainbow trout gill cells, exposing them to copper concentration (2 ± 1 $\mu g.L^{-1}$) at pH 6 and 7, and saw a very significant increase (25-35 times) in the formation of reactive oxygen species. The technique they used to assess the generation of ROS was the same as used in this work, showing that the amount of ROS through H_2DCF-DA is increased when cells are exposed to copper.

The occurrence of xenobiotic contaminants, such as metal ions, in the marine environment is highly harmful to the biological integrity as well as the function of marine organisms. Many of these pollutants are chemicals capable of causing damage to DNA, usually through oxidative stress. Benzo[a]pyrene, for example, increases the amount of ROS which leads to formation, directly or indirectly, of DNA adducts, resulting in genetic chain breaks (Mitchelmore &

Hyatt, 2004; Sarkar et al, 2008). Bopp and Collaborators (2008), in addition to investigating the formation of reactive oxygen species in gill cells exposed to copper, also performed comet assays, to analyze DNA integrity in cells. The results obtained show an increase in DNA molecule damage of 3-5 times higher as compared to the controls in those cells at pH 7, corroborating what was seen in this work, where the exhibition of the cells of the anemone to copper increases the fragmentation of DNA, identified by size and percentage of DNA in the tails of our comet analysis. Our results agree with the premise that copper and the presence of ROS are essential factors for the DNA damage, with the reactive oxygen species performing a crucial role in the process of DNA degradation, causing direct damage through oxidation of the DNA molecule, or indirect, causing damage to lipids and proteins, subsequently leading to genotoxic damage (Almeida *et al.*, 2007; Bopp *et al.*, 2008; Itziou *et al.*, 2011; Mitchelmore & Chipman, 1998; Sandrini *et al.*, 2009; Schwarz *et al.*, 2013). In this paper we chose to highlight primarily the size of tail and the percentage of DNA present our reasoning being that highlighting these separate results (rather than using the tail moment) would better show the type of damage caused. In the work of Mitchelmore and Chipman (1998), the authors comment that simple breaks in DNA chain would be best highlighted by the percentage of DNA presented in the tail. And, generally, damage caused by oxidative stress directly over the DNA molecule, generates breaks in this structure. However, many studies show readers the results in the form of tail moment (Bopp *et al.*, 2008; Valverde & Rojas, 2009). Schwarz and Collaborators (2013), exposed coral cells to copper (0-50 $\mu g.L^{-1}$) and performed comet assay, noting an increase in the percentage of DNA present in tail of almost 40% in those cells exposed to 30 $\mu g.L^{-1}$. Mitchelmore & Hyatt (2004), used coral cells, exposing them to benzo[a]pyrene, hydrogen peroxide and ethylmethane sulfonate (EMS - mutagen) for 1 hour, demonstrating a DNA damage in the 3 levels analyzed in the Comet assay, specifically tail size, percentage of DNA and the moment of tail.

As well as on the work of Schwarz and Collaborators (2013) it was seen that there is an increase in DNA damage in cells exposed to copper, with a clear increase in the percentage of genetic material in the tail. This work confirms the results seen in our experiments, namely, that copper has the

potential to cause DNA damage, especially in relation to dispersion of the genetic material (larger DNA % than tail size, itself), which seems to show simple breaks in DNA chains. It is noteworthy that the percentage of DNA present in the tail seems to be, among the parameters analyzed at Comet assay, the most sensitive, as it was the analysis that managed to show damage in 6 hours of exposure, even in the copper concentration 7.8 $\mu g.L^{-1}$.

By the results obtained in this work, it is clear that copper, even at low concentrations and even within the limit allowed by Brazilian law, causes stress in anemone cells. This metal exhibits cytotoxicity, leading cells to activate cellular defense mechanisms to some extent (in lower concentration). This defense is not sufficiently effective to prevent an increase of reactive oxygen species and genetic damage, however, when it is not activated, (in the highest concentration) the cytotoxic and oxidative genetic damage, are even greater. As this biological model is little studied, there is a lack of data for comparison and further studies on the physiology of these animals are needed. There is also the need for these same experiments to be repeated *in vivo* for a confirmation of the response of these animals facing the pollutant, so that it can be established that the *in vitro* model is trustworthy as the prerogative of the physiological responses of *Bunodosoma cangicum* anemones.

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Considerações finais

Este trabalho é pioneiro na técnica de cultura primária de células de anêmonas *Bunodosoma cangicum* através de explante de tecido do disco podal, ficando evidente que esta metodologia parece ser segura e eficaz para experimentos *in vitro.*

A exposição de células de anêmonas ao cobre, mesmo dentro do limite permitido pela legislação brasileira, representa uma adversidade pelo qual as anêmonas *B. cangicum* precisam responder. Esse metal causa efeito citotóxico, diminuindo a viabilidade e o número de células nestes organismos, apresentam uma indução do mecanismo MXR, ou seja, há uma ativação das defesas celulares. Além disso há um significativo aumento da quantidade de espécies reativas de oxigênio, que pode ser a causa para o aumento do dano à molécula de DNA, o qual foi evidenciado através do ensaio cometa.

Como este modelo biológico é pouco estudado, mais trabalhos são necessários para responder as questões fisiológicas destes organismos frente às adversidades ambientais, com destaque para a repetição dos mesmos experimentos aqui realizados utilizando o modelo *in vivo*, de modo a confirmar que os resultados aqui obtidos são a maneira como o organismo, como um todo, reage a exposição ao cobre.

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