UNIVERSIDADE ESTADUAL DE MATO GROSSO DO SUL
UNIDADE UNIVERSITÁRIA DE AQUIDAUANA
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA

ORGANISMOS AQUÁTICOS COMO MODELO PARA AVALIAÇÃO ECOTOXICOLÓGICA NO PANTANAL SUL-MATOGROSSENSE

Acadêmica: Mayara Pereira Soares

Aquidauana-MS
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UNIVERSIDADE ESTADUAL DE MATO GROSSO DO SUL
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA
PRODUÇÃO ANIMAL NO CERRADO-PANTANAL

MAYARA PEREIRA SOARES

Dissertação submetida ao Programa de Pós-Graduação em Zootecnia, área de concentração em Produção Animal no Cerrado-Pantanal, como requisito para obtenção do grau de Mestre em Zootecnia.

DISSERTAÇÃO APROVADA EM 28/07/2016.

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Dr. William Marcos da Silva (UFMS)

Dra. Camila Aoki (UFMS)

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RESUMO

O objetivo deste estudo foi avaliar a sensibilidade de *Macrobrachium pantanalense*, espécie de camarão de água doce endêmica do Pantanal, aos efeitos do cobre como composto de referência e cipermetrina por meio da formulação Barrage® que é um defensivo químico amplamente utilizado na região do Pantanal de Mato Grosso do Sul. Adicionalmente, comparamos com outras espécies aquáticas, como o camarão de água doce *M. amazonicum*, o microcrustáceo *Daphnia similis* e peixe de água doce *Danio rerio*. O estudo revelou que a sensibilidade das espécies diminuiu na seguinte ordem relativamente à exposição ao Cu: *D. similis* (48h-50 LC 51,22 µg L^{-1})> *M. pantanalense* > *D. rerio* > *M. amazonicum* (120h LC-50 5,96 mg L^{-1}). A sensibilidade das espécies quanto a cipermetrina, diminuiu relativamente na seguinte ordem: *M. pantanalense* (120h-LC 50 0,063 mg L^{-1}) ≥ *M. amazonicum* > *D. similis* > *D. rerio* (144h LC-50 1,680 mg L^{-1}). Os resultados deste estudo demonstraram a alta toxicidade da cipermetrina para as espécies de camarões testados, particularmente a endêmica *M. pantanalense*, destacando a sensibilidade das espécies do Pantanal a produtos químicos de origem antropogênica. Desta maneira, levanta uma grande preocupação sobre os efeitos dos defensivos químicos nesse bioma e a necessidade de realizar estudos de impacto ecológico com espécies locais, como complemento para as espécies modelo atualmente utilizadas.

Palavras-chave**:** Cipermetrina; Cobre; *Danio rerio*; *Daphnia similis*; *Macrobrachium amazonicum.*

ABSTRACT

The goal of this study was to assess the sensitivity of *Macrobrachium pantanalense*, a freshwater shrimp species endemic of Pantanal, to copper as reference compound and to cypermethrin through the formulation Barrage ®, a pesticide widely used in Pantanal (region of Mato Grosso do Sul). Additionally, we compared the sensitivity of *M. pantanalense* to other aquatic species, as the freshwater shrimp *M. amazonicum*, the microcrustacean *Daphnia similis* and the freshwater fish *Danio rerio*. This study revealed that species sensitivity decreased in the following order concerning exposure to Cu: *D. similis* (48h-50 LC 51,22 µg L^{-1})> *M. pantanalense* > *D. rerio* > *M. amazonicum* (120h LC-50 5,96 mg L^{-1}). Concerning cypermethrin, species sensitivity decreased in the following order: *M. pantanalense* (120h-LC 50 0,063 mg L^{-1}) ≥ *M. amazonicum* > *D. similis* > *D. rerio* (144h LC-50 1,680 mg L^{-1}). The results of the present study demonstrated the high demonstrated the high toxicity of cypermethrin for the tested shrimp species, particularly the endemic *M. pantanalense*, highlighting the sensitivity of the Pantanal species to anthropogenic chemicals. Thus, this study raises concern about the effects of chemicals on this biome and the need to perform ecological impact studies with local species as complement to the model species currently used.

Keywords: Cypermethrin; copper; *Danio rerio*; *Daphnia similis; Macrobrachium amazonicum*

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

O Pantanal constitui um dos maiores territórios alagados e é um ecossistema que tem importante papel na conservação da biodiversidade (WWF, 2015). Ecossistema é definido como um conjunto de organismos vivos, plantas ou animais associados a elementos não vivos, como ar, água e solo, assim como a interação entre estes e com o ambiente (SMITH; SMITH, 2012). De acordo com Oliveira e Marandino (2011) podemos encontrar diferentes definições de biodiversidade na literatura que abordam tópicos sobre diversidade genética, de espécie e de ecossistema, sendo mais utilizada a definição diversidade de espécies em comparação com as demais.

Simultaneamente com os primeiros levantamentos sistemáticos de biodiversidade e estudo aprofundado dos ecossistemas, surge a preocupação com os efeitos do desenvolvimento humano sobre o frágil equilíbrio destes (GOMES; BARIZON, 2014). O desenvolvimento humano de maneira não responsável como a expansão da pecuária e agricultura, a exploração de plantações de soja, cana de açúcar e eucalipto podem gerar impactos ambientais no Pantanal (WWF, 2015).

No que diz respeito à atividade agrícola de um modo geral, ainda há grande dependência da utilização de defensivos agrícolas para o controle de pragas. Os resíduos destes defensivos agrícolas são muitas vezes arrastados até os corpos hídricos, tendo efeitos deletérios nos organismos aquáticos que ali vivem, colocando em risco toda a cadeia trófica (GOMES; BARIZON, 2014).

O fenômeno descrito anteriormente pode atingir o Pantanal, sendo um bioma considerado pela UNESCO como Patrimônio Natural Mundial e Reserva da Biosfera (WWF, 2015). Até o momento, foram efetuados poucos estudos ecotoxicológicos que analisam estes efeitos focando o Pantanal de Mato Grosso do Sul. No presente estudo efetuou-se a avaliação ecotoxicológica de modo a analisar o efeito da atividade agrícola sobre a espécie de camarão de água doce *Macrobrachium pantanalense* endêmico do Pantanal de Mato grosso do Sul.

O *M. pantanalense* é uma espécie descrita recentemente por Dos Santos; Hayd e Anger (2013). Esses autores, afirmam que os espécimes são comumente encontrados em ambientes lênticos, próximos às margens dos rios e associados aos bancos de macrófitas que lhes fornecem abrigo e alimentação.

Espera-se com os resultados desse estudo oferecer subsídios científicos que contribuam para o uso sustentável de defensivos químicos no pantanal, bem como o emprego do camarão do Pantanal como organismo modelo para avaliação ecotoxicológica.

O trabalho desenvolvido está dividido em três capítulos: capítulo 1 são explanadas as considerações gerais, onde abordamos tópicos como Pantanal, ameaças ecológicas no Pantanal, estudo ecotoxicológico e a espécie foco - o camarão *M. pantanalense*; Capítulo 2, o manuscrito mostrando os resultados dos experimentos e Capítulo 3 com as conclusões obtidas nesse estudo.

Desta forma, o objetivo deste estudo é analisar o uso de um crustáceo endêmico do Pantanal, o camarão *M. pantanalense*, como organismo modelo para avaliação da problemática dos efeitos do uso de defensivos agrícolas no Pantanal.

2. REVISÃO DE LITERATURA

2.1 O PANTANAL

O Pantanal está localizado no centro da América do Sul, inserido na bacia hidrográfica do alto Paraguai e possui uma extensão de 361.666 km^2 distribuídos no Brasil com 138.183 km^2 (35,36% no estado de Mato Grosso e 64,64% no Mato Grosso do Sul), Bolívia e Paraguai (Fig 1). Além disso, este é constituído por 11 sub-regiões (Cáceres, Poconé, Barrão de Melgaço, Paraguai, Paiaguás, Nhecolândia, Abobral, Aquidauana, Miranda, Nabileque e Porto Murtinho) de acordo com a drenagem dos grandes rios que formam a bacia hidrográfica do rio Paraguai, por ser uma imensa área deprimida que drena os planaltos circunvizinhos (DA SILVA; ABDON, 1998).

Figura 1 - Distribuição do Pantanal na América do Sul (DAMASCENO JUNIOR, 2014).

O bioma Pantanal é caracterizado por uma superfície alagada, sendo uma das maiores áreas úmidas do planeta. Organizações ambientais como a “WWF” e “Biodiversity support programme” enfatizam a necessidade de preservação desse ecossistema devido a características de seus recursos naturais, como a fauna e a hidrologia da região. Por isso, em 1988 o Pantanal foi considerado pela constituição brasileira National Heritage e em 1993 a UNESCO declarou o Pantanal um Patrimônio Mundial (DO RIO, 2011; WWF, 2015).

No Pantanal ocorrem regimes anuais hídricos conhecidos como pulsos de inundações, responsáveis pela produtividade e biodiversidade da região. Estes regimes hídricos fazem com que a cada ano entre os meses de novembro a março ocorram inundações nas planícies pantaneiras e entre maio a outubro ocorra o período de seca nas mesmas. Esse ciclo pode variar de acordo com a região do Pantanal e com as variações climáticas de cada ano. As inundações favorecem a decomposição das vegetações submersas no início da enchente e enriquece a água com matéria orgânica. Posteriormente, toda essa água é transportada para o leito dos rios e lagoas marginais (CALHEIROS, 2003; POTT; POTT, 2004; JUNK et al., 2006; ALPIZAR; CARLSSON; NARANJO, 2011; ALHO et al., 2011; DAMASCENO JUNIOR et al., 2014).

Diversos autores estudaram este fenômeno natural que ocorre no Pantanal (CALHEIROS, 2003; RESENDE, 2008; ALHO et al., 2011; DE OLIVEIRA; CALHEIROS; PADOVANI, 2013). Este tipo de fenômeno oferece benefícios tais como: retenção de água e sedimentos, purificação das águas, estabilização do clima na região, manutenção da biodiversidade e da qualidade de vida para as populações locais (JUNK et al., 2006; WWF, 2015).

O pulso de inundação no Pantanal é um fenômeno tão importante que a perda desse processo pode acarretar em decréscimo da biodiversidade nos ecossistemas aquáticos pela indisponibilidade de nutrientes. Um exemplo deste fenômeno foi estudado por Galdino e Vieira (2006) no Rio Taquari, que está permanentemente inundado em uma área estimada de 5000 km^2, devido ao assoreamento e soerguimento do seu leito. Esse é um processo ecológico chave que contribui para uma grande riqueza em vegetação e alta produtividade do sistema, que por sua vez, suporta uma fauna exuberante constituída por 263 espécies de peixes, 41 de anfíbios, 113 de répteis, 463 de aves, 132 de mamíferos e uma diversidade considerável de camarões de água doce (WILLINK, 2000; MURPHY; AUSTIN, 2005; ALHO, 2008; RESENDE, 2008).

Espera-se, para esta área, um aumento de espécies identificadas, uma vez que estão sendo realizados diversos levantamentos sistemáticos e de biodiversidade (WILLINK, 2000; JUNK et al., 2006; DOS SANTOS; HAYD; ANGER, 2013). Existe também uma crescente preocupação com os efeitos do progresso das atividades humanas sobre o funcionamento deste particular e único ecossistema (CALHEIROS; OLIVEIRA; DOLORES, 2006; GOMES; BARIZON, 2014).

2.2 AMEAÇAS ECOLÓGICAS NO PANTANAL

O progresso das atividades humanas no Pantanal se prende às seguintes atividades: agricultura, pecuária, caça, pesca, extração de minério e turismo (POTT; POTT, 2004; ROSS; SANCHES, 2006). Com a expansão da atividade agrícola e pecuária nesta região, áreas de matas e cerrados dão lugar ao cultivo de soja, arroz, milho, trigo, feijão, algodão e pastagens (GALDINO; VIEIRA, 2006). Essas atividades são caracterizadas pelo uso de defensivos químicos e variados princípios ativos para garantir uma boa produtividade, podendo causar sérios problemas de contaminação de águas superficiais e subterrâneas (GALDINO; VIEIRA, 2006; ROSS; SANCHES, 2006; ALPIZAR; CARLSSON; NARANJO, 2011).

Os resíduos de defensivos químicos podem alcançar o meio aquático por duas formas: transporte pelas águas das chuvas sendo carreados das áreas onde são aplicados (por exemplo, culturas de milho ou soja) ou por contato direto com o meio aquático, como ocorre no plantio de arroz (produzido em áreas de alagamento) (ROSS; SANCHES, 2006; ALHO, 2008). Assim, o uso de defensivos químicos no Pantanal e seu entorno traz uma série de preocupações devido ao funcionamento e a diversidade deste Bioma. Prevê-se que esta prática antropogênica pode culminar em grandes desequilíbrios ecológicos, embora existam ainda poucos estudos sobre a toxicidade dos defensivos químicos para organismos aquáticos residentes nesta região (DORES, 2015). Alguns estudos de monitoramento relatam a presença de resíduos de defensivos químicos no nordeste do Pantanal em 68% das amostras de água de superfície (n = 139), 87% das amostras de água da chuva (n = 91) e 62% das amostras de sedimentos (n = 26) (LAABS et al., 2002).

Um dos defensivos químicos mais utilizados nas atividades agrícolas do Estado de Mato Grosso do Sul é o BARRAGE®, um concentrado emulsionável cujo princípio ativo é a cipermetrina (alfa-ciano-3-fenoxibenzil-2,2-dimetil-3-(2,2-diclorovinil) - ciclopropano carboxilato). Está formulação contém 150 gramas de cipermetrina por litro, sendo comercializada pela empresa ZOETIS - FORT DODGE – Brasil.

O BARRAGE® é utilizado por meio de pulverização para aplicações agrícolas, bem como em aplicações para fins domésticos (controle de carrapatos e insetos), assim como em bovinos para controle de moscas ou carrapatos (BARROS, 1992; NPTN, 1998; GOMES; KOLLER; BARROS, 2011). A cipermetrina atua como uma neurotoxina afetando rapidamente o sistema nervoso central dos insetos, sendo altamente tóxico para invertebrados (por exemplo insetos aquáticos e abelhas), ou vertebrados (por exemplo peixes) (NPTN, 1998).

Em estudos realizados no Pantanal de Mato Grosso do Sul, Calheiros, Dolores e Oliveira (2006) encontraram cipermetrina em amostras de águas superficiais (principalmante nos efluentes do rio Paraguai) e também em sedimentos em outras áreas do estado de Mato Grosso do Sul. Resíduos de cipermetrina foram também encontrados em cinco amostras (n = 104) de água da chuva em Lucas do Rio Verde (Mato Grosso, Brasil) em concentrações entre 0,02 e 0,52 mg L^{-1} (MOREIRA et al*.,* 2012). Embora as concentrações detectadas sejam baixas, uma análise de risco ambiental é essencial devido a elevada toxicidade da cipermetrina para os organismos aquáticos e a elevada fragilidade do bioma Pantanal (GOMES; BARIZON, 2014).

Torna-se necessário avaliar o impacto da cipermetrina para os organismos endêmicos do Pantanal uma vez que outros estudos apontam para altos valores de toxicidade mesmo em baixas concentrações como:, 0,26 µg L^{-1} (48h-LC_{50} para *Daphnia magna* (WALKER; KEITH, 1992)), 0,05 µg L^{-1} (96h-

LC_{50} para peixe-zebra (SATHYA et al., 2014)) e 0,019 µg L^{-1} (96h-LC_{50} para o camarão *Paratya australiensis* (KUMAR et al., 2010)). Dada a sua elevada toxicidade, a concentração máxima permitida de cipermetrina em água é de 0,09 ng L^{-1} (CROMMENTUIJN et al., 2000).

2.4 ECOTOXICOLOGIA

Ecotoxicologia é o termo utilizado para o estudo que retrata os prejuízos dos químicos no ambiente, a compreensão e previsão de efeitos dos poluentes em seres vivos e comunidades naturais, bem como o estudo da interação químicos, seres vivos e ambiente. (KNIE; LOPES, 2004; ZAGATTO; BERTOLETTI, 2008).

Os estudos ecotoxicológicos avaliam os efeitos agudos e crônicos, produzidos por substâncias químicas, afim de estimar o quanto são tóxicos e como desenvolvem seus danos (ZAGATTO; BERTOLETTI, 2008). Em estudos realizados com peixe zebra expostos ao cobre foram observadas algumas anomalias tais como: alterações comportamentais, aumento da incidência de deformidades, efeitos na sobrevivência, retardo no tempo de eclosão, efeitos sobre o batimento cardíaco e edema de pericárdio (JOHNSON; CAREW; SLOMAN, 2007; BAI et al., 2010; HUA et al., 2014). Estudos relatam também, redução no consumo alimentar de juvenis da espécie *Penaeus monodon* expostos ao Cu e menor comprimento da carapaça para animais submetidos a concentrações de Cu na ordem dos 0,90 mg L^{-1} e por vezes superiores (CHEN; LIN, 2001).

Os testes ecotoxicológicos dividem-se em duas categorias: agudos e crônicos. Os agudos, são testes a curto prazo, detectam os efeitos súbitos e geralmente irreparáveis, já os crônicos são de longo prazo, avaliando-se danos que se manifestam após um tempo maior (KNIE; LOPES, 2004). Uma medida utilizada na avaliação destes testes é a Concentração Letal (CL_x) que corresponde à concentração conducente a *x*% de mortalidade para os organismos submetidos ao contato com determinado composto por um período de exposição. Assim, o CL_{50} será a concentração de químico que provocará a morte a 50% dos organismos teste durante o período de observação (ZAGATTO; BERTOLETTI, 2008).

Vários organismos aquáticos podem ser utilizados em testes de toxicidade, sendo designados como “organismos modelo”. Estes devem ter determinadas características tais como: possibilidade de serem facilmente cultiváveis ou mantidos em laboratório e sensibilidade equilibrada que permitem reagir seguramente só aos efeitos tóxicos reais, pois, organismos hipersensíveis podem provocar resultados falsos causados por fenômenos marginais, como por exemplo, mudanças na temperatura. Organismos que apresentam fortes mecanismos fisiológicos de defesa também são inadequados para este tipo de estudos pois podem apresentar resultados subestimados (KNIE; LOPES, 2004; ZAGATTO; BERTOLETTI, 2008). Tendo em conta todos estes fatores a escolha do organismo modelo em uma avaliação de risco é um passo fundamental. Muitos estudos apontam para uma maior sensibilidade de organismos endêmicos, uma vez que o uso de organismos modelo mais generalizados podem em certos casos resultar em uma estimativa equivocada do risco ambiental (PANDRANGI et al., 1995; ZAGATTO; BERTOLETTI, 2008).

Estudos utilizando espécies do gênero *Macrobrachium* (*M. amazonicum*, *M. rosenbergii* e *M. olfersii*) em testes toxicológicos vem sendo realizados por diversos autores (DE MEDEIROS et al., 2001; CAMACHO-SÁNCHEZ; GAMBOA-DELGADO, 2006; CAMACHO-SÁNCHEZ, 2007; CHANG; RAHMAWATY; CHANG, 2013; REVATHI et al., 2014; FERREIRA et al., 2015; BARBIERI et al., 2016;), no entanto, estas espécies não são endêmicas da região pantaneira e apresentam ciclo de vida e biologia diferenciado, com fases do ciclo larval ocorrendo em regiões de estuário. Estas espécies, após se transformarem em pós-larvas, começam a migrar para o interior dos rios tendo seus demais estágios de desenvolvimento em água doce (MELO, 2003; MURPHY; AUSTIN, 2005; DE GRAVE; CAI; ANKER, 2008). Sendo assim, a utilização de uma espécie nativa para avaliação de toxicidade possibilita maior acurácia e confiança nos resultados das avaliações ecotoxicológicas.

A ecotoxicologia é, assim, uma ferramenta muito importante para se conhecer os efeitos dos defensivos químicos, utilizados na agropecuária, que podem atingir o ambiente e, por sua vez, as comunidades aquáticas. De acordo com Gomes e Barizon (2014) a avaliação de risco ambiental é indispensável para apoiar e promover medidas de mitigação dos efeitos tóxicos sobre o meio ambiente. Desta maneira, selecionou-se a utilização do *M. pantanalense* como organismo modelo em avaliação de risco ambiental no Pantanal de Mato Grosso do Sul acreditando na sua viabilidade, pois além de se tratar de uma espécie endêmica apresenta características desejáveis para estudos tal como a facilidade de cultivo em laboratório.

2.5 *Macrobrachium pantanalense*

A espécie de camarão que ocorre no Pantanal *M. pantanalense* foi durante muito tempo considerada como *M. amazonicum*, porém recentemente foi distinguido e identificado como uma nova espécie, sendo as principais diferenças encontradas na morfologia e nos padrões de cor de ambos os sexos (Fig 2). *Macrobrachium pantanalense* é transparente, apresenta manchas castanhas em todo o corpo e tamanho reduzido (DOS SANTOS; HAYD; ANGER, 2013).

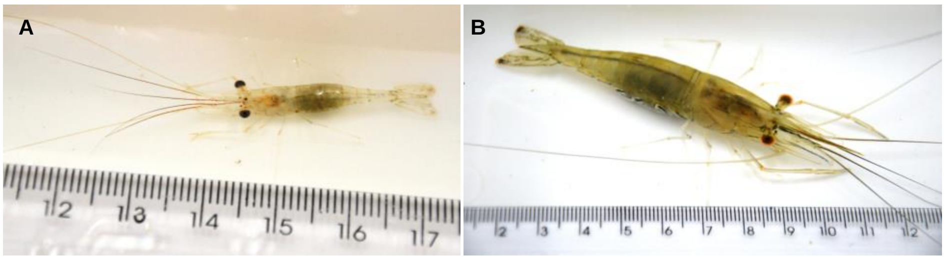

Figura 2 - Fêmea ovígera de *Macrobrachium pantanalense* (A) e *Macrobrachium amazonicum* (B) (Fonte: pessoal)

Esta espécie apresenta dimorfismo sexual, os machos são menores que as fêmeas (HAYD; ANGER, 2013). Estes autores indicam que os machos iniciam a atividade sexual com tamanho corporal próximo de 19 mm, e para as fêmeas o início da maturidade é observado pelo tamanho mínimo corporal das fêmeas ovígeras (29,8 mm).

O ciclo de vida é dividido em ovo, fase larval, juvenil e adulto. A fecundidade das fêmeas depende do seu tamanho podendo chegar a um limite próximo a 676 ovos (HAYD; ANGER, 2013; VERCESI; HAYD, 2015).

O desenvolvimento embrionário das larvas dura cerca de 19 a 23 dias e estas passam por 11 estágios larvais (fases de zoea), apresentando nesse período fototaxia positiva e hábito alimentar planctônico (Fig.3). Posterior a este estágio ocorre metamorfose chegando a fase de pós-larva, quando começam a apresentar hábito alimentar bentônico e conseguem nadar livremente pela lâmina d’água (HAYD; ANGER, 2013).

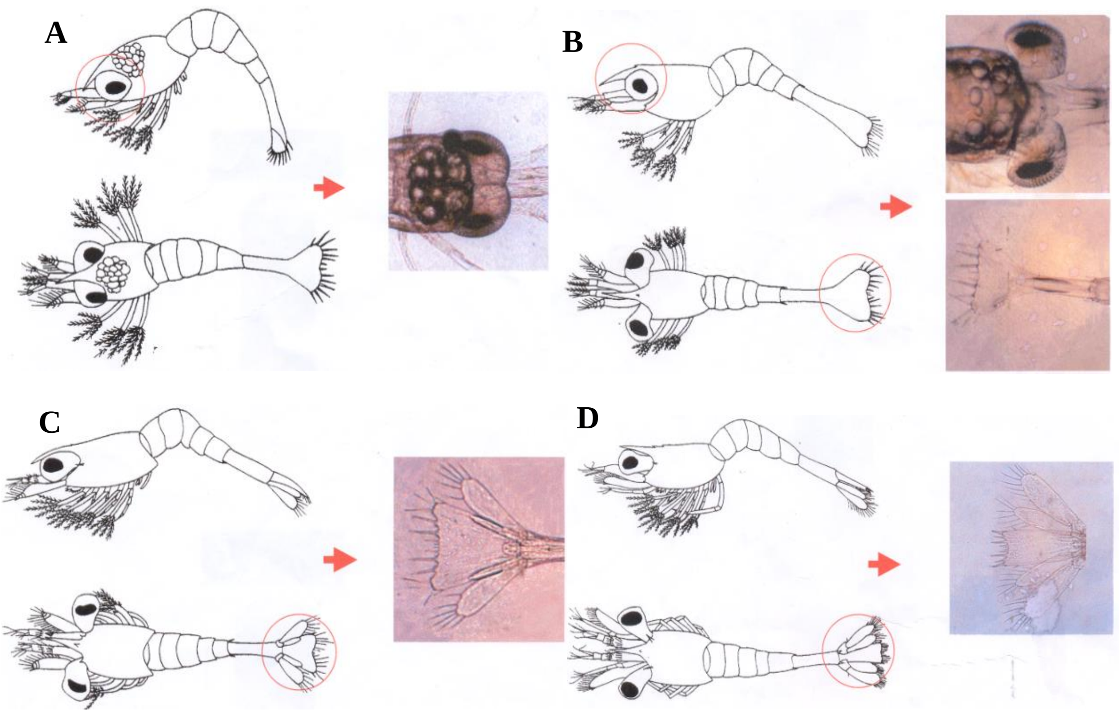

Figura 3 - Fases de zoea de Macrobrachium: zoea I, olhos sésseis (A); zoea II, olhos pedunculados; urópodos ausentes (ausência de endopodito e exopodito), apenas o télson aparece no último segmento (B); zoea III, urópodos constituídos por exopoditos desenvolvidos, com cerdas e endopoditos rudimentares, nus, sobre o télson (C); zoea IV, exopoditos e endopoditos dos urópodos desenvolvidos e com cerdas (D) (adaptado de VEGA PEREZ, 1984).

Esta espécie tem desenvolvimento larval e adulto em água doce, pois habita águas interiores (HAYD; ANGER, 2013), porém, estudos mostram que a salinidade pode reduzir o período de tempo do desenvolvimento larval dessa espécie quando cultivadas em laboratório. Vercesi (2014) verificou que quando cultivadas em inanição, as larvas de *M. pantanalense* apresentam maior sobrevivência em salinidade 5.

Por ser uma espécie endêmica e possuir cultivo relativamente fácil é possível validar o *M. pantanalense* como organismo modelo para estudos em ecotoxicologia, uma vez que esta espécie pode representar a comunidade de crustáceos endêmicos do Pantanal. A espécie está distribuída pela Bacia do Rio Paraguai, possuindo assim um papel chave nas cadeias tróficas deste ecossistema.

A validação dos estudos será possível com o desenvolvimento de testes ecotoxicológicos com esta espécie e com a comparação dos resultados com organismos semelhantes, tais como *M. amazonicum*, uma espécie amplamente distribuída na América do Sul (Fig 4) e com espécies aquáticas frequentemente utilizadas em testes ecotoxicológicos como Dáfnias e peixe-zebra (ZAGATTO; BERTOLETTI, 2008).

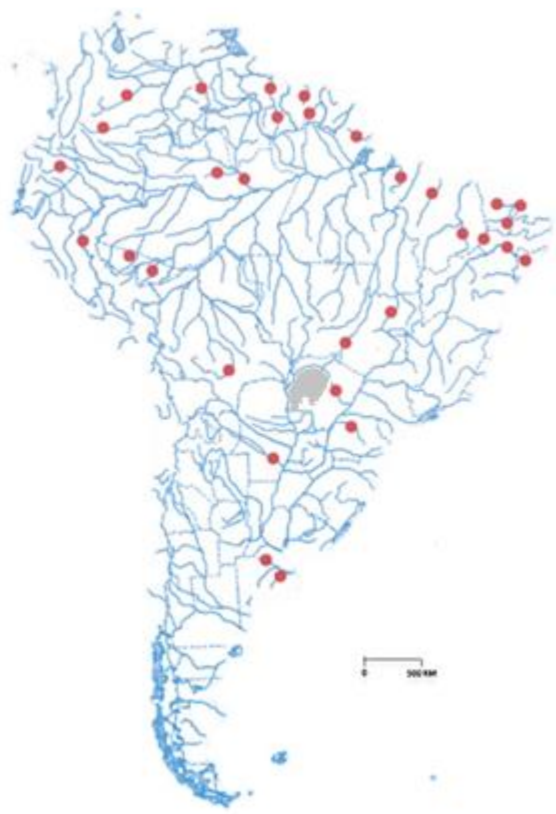

Figura 4**-** Distribuição geográfica de *Macrobrachium pantanalense* ( ) e *Macrobrachium amazonicum* ( ) (adaptado de MELO, 2003).

3. OBJETIVOS

3.1 Objetivo geral

Avaliar os efeitos da toxicidade dos defensivos agrícolas usados no Pantanal utilizando larvas do camarão de água doce *Macrobrachium pantanalense* como organismo modelo.

3.2 Objetivos específicos

a) Avaliar a sensibilidade de *Macrobrachium pantanalense* como organismo modelo utilizando um composto de referência (sulfato de cobre) em comparação com *M. amazonicum*; *Daphnia similis* e *Danio rerio*.

b) Avaliar o efeito tóxico do defensivo agrícola usado no Pantanal (a cipermetrina) em larvas de *M. pantanalense* e comparar esse efeito com *M. amazonicum*; *D. similis* e *D. rerio*;

c) Avaliar a adequação de *M. pantanalense* como organismo modelo de poluição aquática do Pantanal.

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CAPITULO 2- CAMARÃO ENDÊMICO *Macrobrachium pantanalense* COMO UMA ESPÉCIE DE TESTE PARA AVALIAR A POTENCIAL CONTAMINAÇÃO POR PESTICIDAS NO PANTANAL (BRASIL)

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Endemic shrimp *Macrobrachium pantanalense* as a test species to assess potential contamination by pesticides in Pantanal (Brazil)

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Abstract

Pantanal is a biome characterized by an extraordinary diversity and abundance of wildlife and houses several endemic species such as the freshwater shrimp *Macrobrachium pantanalense*. However, the increase in agriculture and husbandry activities in the region has contributed with residues of pesticides reaching aquatic systems. The main objective of this study is to assess the sensitivity of the endemic shrimp *M. pantanalense* compared with other freshwater species: the shrimp *M. amazonicum* (a closely related species), the crustacean *Daphnia similis* and the fish *Danio rerio*. The sensitivity of these organisms was assessed through acute exposure to copper and cypermethrin (through the formulation Barrage®, widely used in Pantanal). For copper the species sensitivity decreased in the following order: *M. pantanalense* (96h-LC_{50} 3.24 µg/L) > *D. similis* > *D. rerio* > *M. amazonicum* (120h-LC_{50} 2.88 mg/L). Copper caused reduced length of shrimps and zebrafish and reduced heartbeat of zebrafish embryos. For CYP the species sensitivity decreased in the following order: *M. pantanalense* (96h-LC_{50} 0.05 µg/L) > *M. amazonicum* > *D. similis* > *D. rerio* (144h-LC_{50} 1.68 mg/L). Major effects of CYP included reduced length of shrimps and zebrafish, as well as early hatching and increased incidence of developmental deformities in zebrafish embryos. This study highlights the importance of using endemic species for risk evaluations in sensitive biomes such as Pantanal. Moreover, it emphasizes the importance of testing pesticides toxicity as commercial formulations. Furthermore, we suggest that the endemic shrimp species *M. pantanalense* can be successfully used as a test species in ecotoxicology.

Keywords: Barrage®; cypermethrin; copper sulphate; *Macrobrachium amazonicum*; *Danio rerio; Daphnia similis*

1. Introduction

Pantanal is a biome characterized by an extensive floodplain (140000 km^2) which is located approximately between 16-20º latitude South and 55-58º longitude West, in western Brazil and Bolivia (Junk et al., 2006). It is known for its extraordinary diversity and abundance of wildlife and is considered a refuge for many threatened or endangered species (Junk et al., 2006), constituting a Biosphere reserve. However, this fragile biome is threatened by deforestation, illegal hunting and fishing and, expanding agriculture and livestock (Alho and Vieira, 1997). Moreover, the increase in agricultural and livestock farming in Pantanal has been sustained by the use of pesticides to ensure good productivity which raises concerns regarding the deterioration of the environmental quality of the biome. Indeed, a monitoring study in northeastern Pantanal identified pesticide residues in 68% of surface water samples (n=139), 87% of rainwater samples (n=91) and 62% of sediment samples (n=26) (Laabs et al., 2002). The insecticide cypermethrin (CYP) can be found among the pesticides detected in the water samples. This compound was detected in surface water (main tributaries of the Paraguay river) and sediment in Mato Grosso do Sul state (Brazil) (Calheiros et al., 2006). CYP residues were also found in five samples (n=104) of rainwater in Lucas do Rio Verde (Mato Grosso state, Brazil) in concentrations between 0.02 and 0.52 µg/L (Moreira et al., 2012). Although the concentrations detected are low, an environmental risk analysis is essential due to both the high toxicity of CYP to aquatic organisms and the high fragility of the biome Pantanal (Gomes and Barizon, 2014).

CYP, a synthetic pyrethroid, is the active ingredient of Barrage®, an emulsifiable concentrate widely used in the Pantanal region as insecticide, for agricultural applications, for domestic purposes (control of ticks and insects in the houses) and for fleas and ticks control in cattle (Barros, 1992; Gomes et al., 2011; NPTN, 1998). CYP acts as a neurotoxin, rapidly affecting the central nervous system of insects being highly toxic to bees, aquatic insects, crustaceans and fish (Keith and Walker, 1992; NPTN, 1998). For example, toxicity values as low as 0.26 µg/L [48h-LC_{50} for *Daphnia magna* (Keith and Walker, 1992)], 0.05 µg/L [96h-LC_{50} for zebrafish (Sathya et al., 2014)] and 0.019 µg/L [96h-LC_{50} for the shrimp *Paratya australiensis* (Kumar et al., 2010)] were reported. Given its high toxicity, the maximum permissible concentration of CYP in the water is 0.09 ng/L (Crommentuijn et al., 2000).

Pantanal holds a high diversity of aquatic animals. In particular, there is a substantial diversity of freshwater shrimps of the genus *Macrobrachium* (Crustacea: Decapoda: Caridea: Palemonidae) (Murphy and Austin, 2005). Moreover, the number of *Macrobrachium* species is still increasing due to the identification of new species (De Grave et al., 2008). This is the case of *M. pantanalense* (Santos et al., 2013), a freshwater shrimp, endemic of Pantanal, previously confounded with *M. amazonicum*, a shrimp species widely distributed throughout South America (Magalhães, 2003). *M. pantanalense* differ from *M. amazonicum* mainly in size, morphology and color patterns of both males and females (Santos et al., 2013).

There is scarce knowledge on the acute toxicity of pesticides to aquatic organisms in Pantanal (Dores, 2015). Using pesticide formulations for toxicity assessment represents a realistic scenario since pesticides are applied as formulations, which contain not only one or more active ingredients, but also inert ingredients. Previous studies have shown that the toxicity of formulations can be significantly higher or lower than that of the active ingredient (Pereira et al., 2009). On the other hand, using endemic species is crucial for a reliable risk assessment. Many studies (e.g. Pandrangi et al, 1995; Zagatto and Bertoletti, 2014) point to a higher sensitivity of endemic species whereas the use of more generalized model species may in some cases underestimate the environmental risk. However, in tropical regions ecotoxicological assays are frequently carried out with non-native species due to the lack of standardized procedures for this purpose. Thus, the hypothesis of this study is that toxicity evaluation is better achieved using an endemic species.

The main objective of this study was to assess the sensitivity of the endemic freshwater shrimp species *M. pantanalense* compared with other aquatic species. The selected species were the freshwater shrimp *M. amazonicum*, which previously included the species *M. pantanalense*; the crustacean *Daphnia similis,* which is a model species commonly used for toxicity assessment in Brazil (ABNT, 2009) and the fish *Danio rerio* (zebrafish), a model species widely used in ecotoxicology (OECD, 2013). The sensitivity of these organisms was assessed through acute exposure to two chemicals: the pesticide CYP (through the formulation Barrage®), a relevant pesticide in Pantanal due to its extensive use, and copper (as copper sulphate). As a reference compound, copper was included in the study for the establishment of protocols for toxicity testing using *M. pantanalense* (which to the best of our knowledge has not been used in toxicity assays before) and for a more consistent comparison of sensitivity among the selected species. The acute toxicity to larvae of *M. pantanalense* and *M. amazonicum* was determined through evaluation of mortality, growth, number of lipid droplets and delays in larval development; toxicity to *D. similis* was assessed by determining immobilization; and toxicity to larvae of *D. rerio* was assessed by determining mortality, hatching success, heartbeat, abnormalities during the embryo development and final length.

2. Material and Methods

2.1 Chemicals

Copper source was copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; CAS Number: 7758-99-8), bought from Sigma-Aldrich Co (assays with *D. similis* and *D. rerio*) or VETEC – Brazil (assays with *M. pantanalense* and *M. amazonicum*). A stock solution was prepared by dissolving the chemical in Milli-Q water and exposure solutions were prepared by diluting in the appropriate medium for each species. Cypermethrin, ([α-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2-2-dichlorovynil) cyclopropane carboxylic acid]; $C_{22}H_{19}Cl_2NO_3$; CAS Number: 52315-07-8) source was the commercial formulation Barrage®, bought from Zoetis-Fort Dodge (Campinas, SP, Brazil). The formulation Barrage® is a concentrated suspension, emulsifiable, containing 150g of CYP per liter. The stock solution and exposure solutions were prepared by diluting the chemical in the appropriate media for each species.

2.2 Test organisms

Parent shrimp (males and females) of *M. pantanalense* and *M. amazonicum* were kept in a recirculating system, under controlled conditions: temperature was 28 ± 1 ºC, conductivity 0.24 µS/cm, pH 7.5 ± 0.5 and dissolved oxygen equal or above 95 % saturation. Culture water was tap water. A 12h:12h (light:dark) photoperiod cycle was maintained. The parent shrimp were fed twice a day with commercially available artificial diet (Alcon Goldfish Colours Bits, bought from Alcon Pet, SC, Brazil, net protein 32%), fish fillet and chopped carrot. Ovigerous females with embryos in the final stages of development (identified by a higher transparency of the egg membrane that allow the visualization of the embryos' eyes as distinctive black dots) were isolated in a 1 L beaker containing culture water. Females were kept inside a plastic network, allowing the larvae to escape and, thus, preventing cannibalism of the larvae. Larvae were collected and submitted to a disinfection bath with 0.025% of sodium hypochlorite to prevent infection with parasites and fungi. Larvae were kept in culture water complemented with salt (Nutratec) (salinity of 5 and 10 for *M. pantanalense* and *M. amazonicum*, respectively) and placed in a chamber with controlled temperature and photoperiod (TE-401 model, Tecnal, Brazil). Water changes (50-60 %) were done every day. Larvae were fed daily with brine shrimp nauplii newly hatched. Two day old larvae were used in the toxicity assays.

Zebrafish embryos were provided by the zebrafish facility at the Biology Department of University of Aveiro (Portugal). Adult organisms were kept under controlled conditions, in a ZebTEC (Tecniplast, Buguggiate, Italy) recirculating system. Culture water was obtained through reverse osmosis and activated carbon filtration of tap water, complemented with salt ("Instant Ocean Synthetic Sea Salt", Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was 27.0 ± 1 ºC, conductivity 750 ± 50 µS/cm, pH 7.5 ± 0.5 and dissolved oxygen equal or above 95 % saturation. A 12h:12h (light:dark) photoperiod cycle was maintained. Adult fish were fed twice a day with commercially available artificial diet (ZM-400 fish food; Zebrafish Management Ltd, UK) and brine shrimp nauplii. The AB wild type fish strain was used in the experiments. Zebrafish eggs were collected within 30 min after natural mating and rinsed in fish system water. To exclude eggs unfertilized injured or with cleavage irregularities, eggs were screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation).

Daphnia similis was cultured in ASTM hard water (ASTM, 2004) with a standard organic additive (Marinure seaweed extract, The Glenside Group Limited, Scotland, UK) and fed *Pseudokirchneriella subcapitata* ($5x10^5$cells/ml). Culture medium was renewed every other day. Temperature was 20 ± 1 ºC and photoperiod was 16h:8h (light:dark). Neonates (<24h) from third to sixth broods were used in the experiments.

2.3 Toxicity tests

2.3.1 Acute tests with shrimp larvae

Previously to the tests, 2-d old larvae were submitted to a 0.025% sodium hypochlorite bath to prevent infections. Tests were carried out under the same conditions as mentioned for the larvae culture. Tests were performed in polyethylene 6-wells microplates with 5 replicates per treatment. Each replicate consisted of 5 larvae, placed in one well of the microplate with 10 ml of solution. The tested Cu concentrations were 0 (control), 0.25, 0.64, 1.59, 3.97 and 9.94 mg/L for *M. amazonicum*, and 0, 0.01, 0.07, 0.3, 1.25 and 5 mg/L for *M. pantanalense*. The tested CYP concentrations were 0, 0.06, 0.18, 0.55, 1.6 and 5 µg/L for both species. The duration of the exposure was 4 days. However, for *M. amazonicum*, Cu assay was repeated and extended until day 5 in order to achieve mortality levels allowing the determination of a LC_{50} value. Larvae were checked daily for mortality, using a stereomicroscope. Larvae were daily fed with newly hatched *Artemia* and test media was renewed every other day after larvae feeding. At the end of the test larvae were anesthetized with carbonated water and fixed in 70% ethanol for posterior determinations. The following parameters were assessed using a stereomicroscope: delay of the larval development, carapace length and number of internal lipid droplets. The larval development was assessed by identifying the developmental stages zoea I, zoea II, zoea III, zoea IV and zoea V, according to Vega-Pérez (1984). The carapace length was measured over the distance between the end of the rostrum (tip) to the median posterior edge of the carapace (see Fig. S1, Supplementary Material). The number of lipid droplets is a measurement of the energetic reserves of the larvae. Lipid droplets can be observed in the hepatopancreas region of the cephalothorax, representing fat droplets remaining from the egg yolk (Anger and Hayd, 2010). This parameter was only assessed in *M. amazonicum* since the species *M. pantanalense* presented no lipid droplets.

2.3.2 Fish Embryo Toxicity Test (FET)

Three hours post fertilizations (hpf) zebrafish embryos were exposed to several concentrations of Cu (0, 0.125, 0.250, 0.5, 1, 2 and 4 mg/L) or CYP (0, 1, 10, 50, 100, 500 1000, 5000 and 10000 µg/L) in 24-well plates according to the OECD guideline 236 (OECD, 2013). Tests were performed under the same conditions as described for the culture using 3 replicates per treatment. Each replicate consisted in 10 embryos placed individually in the wells with 2 ml of test solution. During 144 h, embryos were observed daily using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). The following endpoints were observed: survival, occurrence of pericardial edema, heartbeat, deformities, hatching success, equilibrium and spasms. Heartbeat was measured after 48 h of exposure by counting the heartbeats during 15 seconds. Larvae length was determined at the end of exposure (144h). In the Cu experiment many embryos did not hatch and, thus, dechorionation was performed to allow measurement of the larvae length. Length was measured through capture of images (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation) and posterior measurement of the larvae length using the program NIS Elements 3.2 (Nikon Corporation). In the CYP experiment, effects on the equilibrium and tremors/spasms were observed. Concerning equilibrium, larvae were classified as “normal”, if the embryo was positioned dorsally and with no signs of unbalance; “unbalance”, if the embryo showed slight signs of unbalance but was dorsally positioned; or “side-ways”, if the embryo was positioned sideways. Fish were considered to have spasms/tremors when they showed sudden and involuntary body movements, or were continuously shaking.

Exposure of zebrafish to CYP resulted in responses not following a dose response curve for the endpoints hatching, length and heartbeat. Thus, a new test was carried out, with slightly different concentrations, to confirm the results. Since the response followed the same pattern as in the first test, data was normalized to the average response of controls and the global results are shown for these endpoints.

2.3.3 Immobilization tests with *D. similis*

Acute tests with *D. similis* followed the OECD guideline N202 (OECD, 2004) and were carried out under the same conditions as mentioned for the cultures, except that they were carried out in the absence of seaweed extract and algae. Five replicates per treatment were used in the test. Each replicate consisted in five neonates (<24h) placed in a glass vial with 50 ml of test solution. Organisms were checked for immobilization after 24 and 48 h of exposure.

2.4 Chemical analysis

At the beginning and end of the tests, samples were collected for posterior chemical analysis. Cu samples were acidified with nitric acid before being analysed by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP–OES, Horiba Jobin Yvon, Activa M model).

Samples for CYP analysis were frozen and posteriorly analysed by liquid/liquid extraction and gas chromatography/mass spectrometry with limit of quantification of 10 ng/L. The extracts were not cleaned up prior GC/MS analyses. Rtx-1 analytical column (15 m length x 0.25 mm ID and 0.1 µm phase thickness) was used for separation. Target analytes were detected with single ion monitoring after negative chemical ionization at Agilent 7890/5975C gas chromatograph/mass spectrometer operated under ChemStation software. Methane was used as reagent gas, isolation window was set to 1 amu for quantification mass 209 m/z and qualification masses 171 and 415 m/z. D6 dimethyl labelled CYP was used as internal standard (Dr. Ehrenstorfer). Cypermethrin was calculated as technical mixture concentration as isomer peaks profile corresponded to this mixture (Fluka, CAS No.: 52315-07-8). In addition, the formulation was analyzed for the presence of solvents using GC/full scan.

2.5 Statistical analysis

Statistical analysis was performed using the software package SigmaPlot (version 12.5, Systat Software Inc., CA, USA). To assess whether Cu or CYP affected each parameter, a one-way ANOVA was performed, followed by the post-hoc Dunnet's test for multiple comparisons relative to the control. For non-normally distributed (Ryan-Joiner test) or heteroscedastic (Levene's test) data, the nonparametric Kruskal-Wallis test and the post-hoc Dunn's test were used. Concerning the delay on developmental stages of the shrimp larvae the $\chi2$-test was used to test for the association of the developmental stages (zoea) and the concentration of Cu and CYP.

The calculation of the LC/EC_{50} values (median lethal/effective concentrations) was performed by fitting the data to dose-response curves using the drc extension package of the statistical program R (Ritz and Streibig, 2005). The significance level for all statistical analyses was 0.05.

3. Results

Results of the chemical analyses are presented in Tables S1 and S2 (Supplementary Material). For both Cu and CYP, there was a good agreement between the nominal and measured concentrations at the beginning of the experiments – in average the difference was less than 15%. For this reason the nominal concentrations were used for data analyses. After 48 and 144h of exposure, the recovery for both Cu and CYP decreased, more pronouncedly for CYP. Indeed, after 48h of exposure only 32% of the initial concentration was found (*D. similis* test), whereas after 144h only 4% of the initial concentration was found (*D. rerio* test).

The chemical analysis also showed that the formulation Barrage® contained a mixture of α and β-isomers of CYP. Moreover, the solvent analysis revealed a mixture of C9 aromatics (trimethylbenzens, methylethylbenzens, propylbenzens, methylstyrens, allylbenzen (2-propenylbenzen)) and a minority of C8 aromatics (dimethylbenzens (xylens)) and C10 aromatics (ethyldimethylbenzens).

3.1 Copper

3.1.1 Acute tests with shrimp larvae

The results of the acute tests of *M. pantanalense* and *M. amazonicum* exposed to Cu are depicted in **Table 1** and **Fig. 1**. Concerning survival, results show that *M. pantanalense* is more sensitive to Cu than *M. amazonicum*. For instance, after 96 h of exposure a LC_{50} value as low as 3.24 μg/L was obtained for *M. pantanalense*, whereas no LC_{50} value could be determined for *M. amazonicum* since mortality levels were below 50% (LC_{50} > 9.96 mg/L) (**Table 1**).

Table 1 – Cu $L(E)C_{10}$ and $L(E)C_{50}$ values (and the respective standard error) for the tested species as well as the models used to fit the data and the respective slope.

Species	Time (h)	Endpoint	$L(E)C_{10}$	$L(E)C_{50}$	Slope	Model
Macrobrachium pantanalense (mg/L)	**96**	Survival	$1.71x10^{-5}$ (0.000)	$3.24x10^{-3}$ (0.004)	0.42 (0.14)	LL.3
Macrobrachium amazonicum (mg/L)	**96**	Survival	n.d.	n.d. (>9.94)		
	120	Survival	0.43 (0.34)	2.88 (0.88)	1.15 (0.38)	LL.3
Danio rerio (mg/L)	**96**	Mortality	1.45 (4.61)	1.87 (0.79)	-4.73 (51.25)	W 1.4
	96	Hatching	0.02 (0.01)	0.17 (0.03)	0.86 (0.22)	W 1.3
	144	Mortality	0.52 (0.12)	1.44 (0.22)	-2.16 (0.62)	LL.4
	144	Hatching	0.20 (0.04)	0.36 (0.03)	9.83 (22.61)	LL.5
Daphnia similis (μg/L)	**24**	Immobilization	44.64 (4.61)	76.3 (3.8)	-4.1 (0.72)	LL.3
	48	Immobilization	31.44 (2.25)	51.22 (1.33)	-4.50 (0.64)	LL.3

LL.3 - log-logistic 3 parameters function; LL.4 - log-logistic 4 parameters function; LL.5 - log-logistic 5 parameters function; W1.3 - Weibul 3 parameters function; W1.4 - Weibul 4 parameters function

Carapace length was significantly reduced by Cu either in *M. pantanalense* (ANOVA, $F_{3, 14}$= 7.2, p<0.006) and *M. amazonicum* (ANOVA, $F_{5, 29}$= 13.98, p<0.001) (**Fig. 1C and D**). The number of lipid droplets in *M. amazonicum* was affected by Cu exposure (Kruskal-Wallis, H=23.76, p<0.001) in spite of the lack of a clear dose-dependent pattern (**Fig. 1D**).

The larval development was delayed for both species (**Fig. 1 E and F**). At the end of the test, control larvae of *M. pantanalense* were in zoea V, and control larvae of *M. amazonicum* were in zoea IV. The delay in the larval development followed a dose-dependent pattern for *M. pantanalense,* but not for *M. amazonicum*. Developmental stage and Cu concentrations were significantly associated for *M. pantanalense* (χ^2=310.32, df=3, p<0.001) and *M. amazonicum* (χ^2=402.67, df=5, p<0.001).

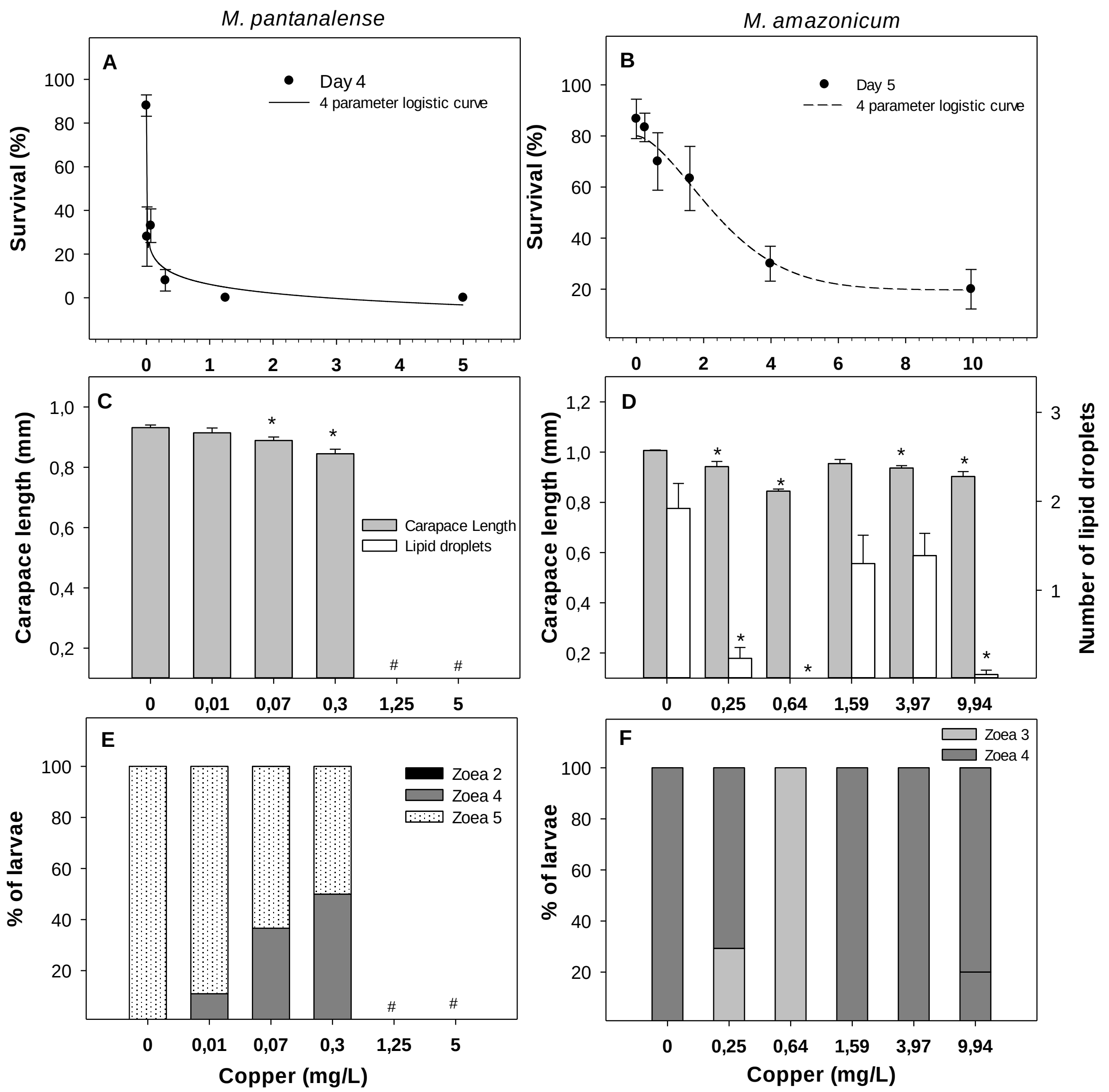

Figure 1 – Cu effects in larvae of *Macrobrachium pantanalense* and *Macrobrachium amazonicum* after 4 d of exposure: survival (A and B) - note that for *Macrobrachium amazonicum*, only survival after 5 d of exposure is presented (graph B); carapace length (C and D); developmental stage (E and F). Values represent means and the error bars represent standard errors. Asterisks denote statistically significant differences relative to the control ($p<0.05$, Dunnett's test for carapace length and Dunn's test for lipid droplets). "#" indicates insufficient data to perform the analysis.

3.1.2 Fish Embryo Toxicity Test

Cu significantly affected survival, hatching rate and occurrence of deformities in zebrafish embryos (**Fig. 2**). The LC_{10} and LC_{50} values are shown in **Table 1**.

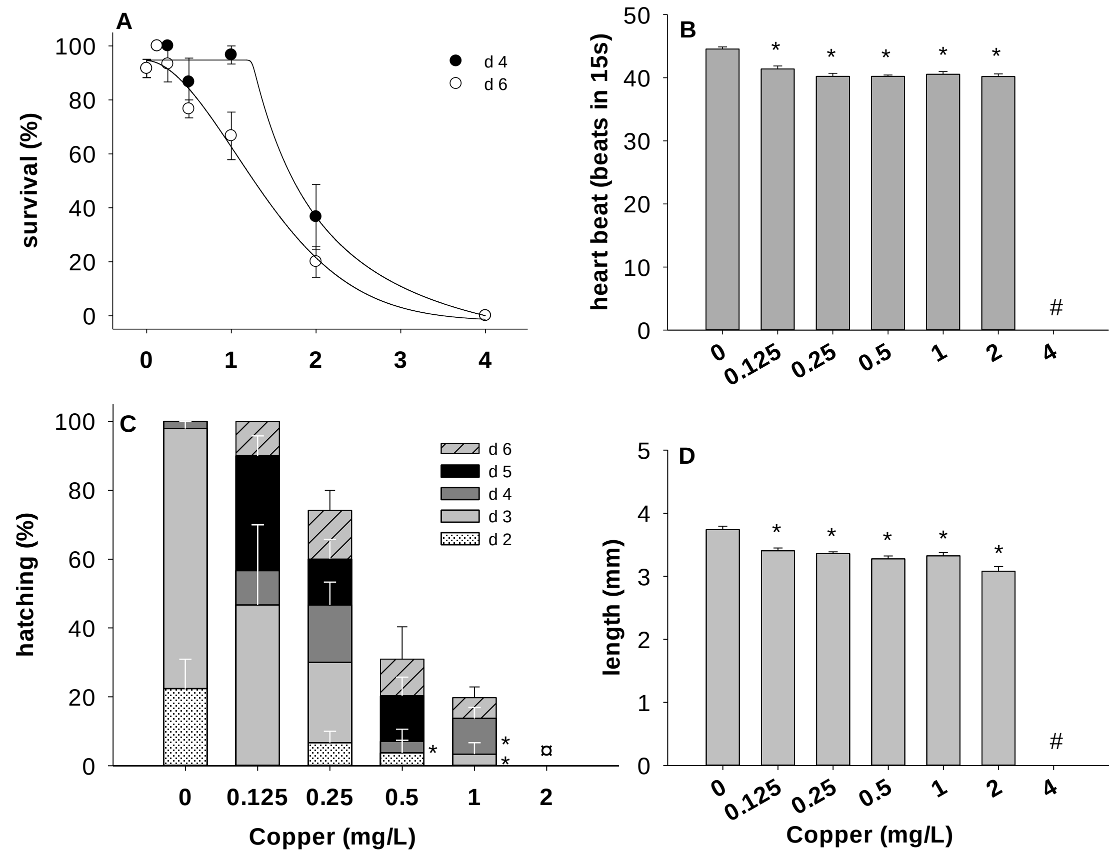

Figure 2 – Cu effects in *Danio rerio* embryos: survival at day 4 and 6 (A); heartbeat measured at 48h for 15 seconds (B); hatching as a percentage of living larvae (C); and larvae length at day 6 (D). Values represent means and the error bars represent standard errors. Asterisks (*) denote statistically significant differences relative to the control ($p<0.05$). Symbol ¤ means that data for 2 mg/L were statistically different from control for d3, d4, d5 and d6. "#" indicates insufficient data to perform the analysis.

Mortality followed a dose response curve, increasing with concentration and with the duration of exposure (**Fig. 2 A**). The survival rate registered in day 1 was constant throughout the test until day 4 and decreased again after 5 days of exposure (**Fig S2** - Supplementary Material).

The hatching success decreased with increasing Cu concentrations, following a dose-response curve (**Fig. 2 C**), both after 3 d (H=14.447, df=5, p=0.013) and 6 d of exposure (H=17.416, df=5, p=0.004). After 3 days of exposure, concentrations as low as 0.5 mg Cu/L caused a significant reduction on the hatching success of zebrafish. In addition, embryos exposed to 2 mg Cu/L showed 0% of hatching success after 6 days of exposure.

Copper exposure also exerted a negative effect on the hearth beat ($F_{5,61}$=25.263, p<0.001) and larvae length ($F_{6,115}$=238.004, p<0.001), with all Cu-exposed embryos showing significantly lower hearth beat and shorter larvae length than control (**Fig. 2 C and D**).

3.1.3 Immobilization test with *D. similis*

Increasing concentrations of Cu caused increased immobilization of daphnids after 24 and 48 h of exposure, following a dose-response curve (Fig. 3). The 48h-EC_{50} was 51.22 µg/L (**Table 2**).

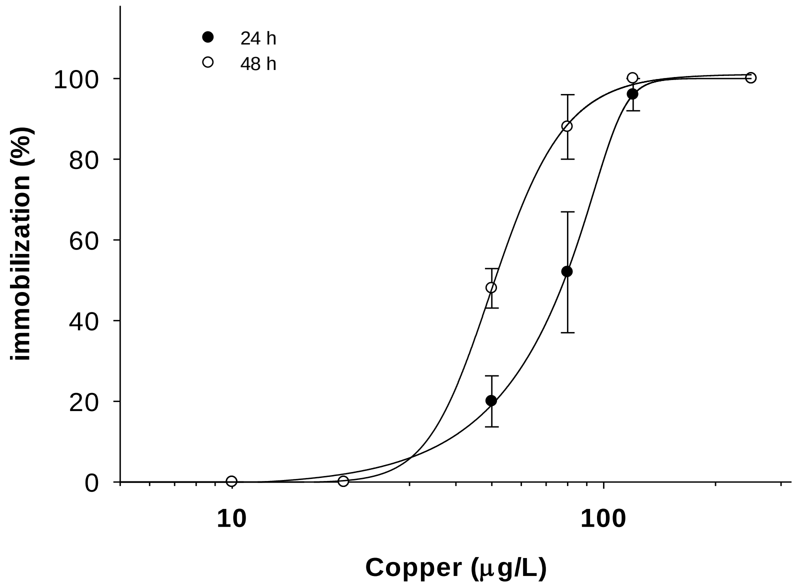

Figure 3 – Immobilization of neonates of *Daphnia similis* after 24 and 48 h of exposure to Cu (symbols represent means and the error bars represent the standard error)

3.2 Cypermethrin

3.2.1 Acute tests with shrimp larvae

CYP, through the formulation Barrage®, caused significant effects on survival, carapace length and development of both shrimp species (**Table 2 and Fig. 4**).

Concerning survival, results show that *M. pantanalense* is more sensitive to CYP than *M. amazonicum* (**Fig. 4 A and B**). Due to high mortality of *M. pantanalense* larvae during the test, carapace length could only be measured at the lowest concentration and no clear effect was detected (U=4.000, p=0.262) (**Fig. 4 C**). The carapace length of *M. amazonicum* was affected by CYP ($F_{3, 13}$= 14.04, p<0.001), being reduced for most of the tested concentrations (0.061, 0.18 and 0.55 µg/L). Lipid droplets were also reduced under exposure to CYP ($F_{3, 13}$= 21.03, p<0.001) **(Fig.4 D**).

Table 2 – Cypermethrin L(E)C_{10} and L(E)C_{50} values (and the respective standard error) for the tested species as well as the models used to fit the data and the respective slope. Cypermethrin was used as the commercial formulation Barrage®.

Species	Time (hours)	Endpoint	L(E)C_{10}	L(E)C_{50}	Slope	Model
Macrobrachium pantanalense (µg/L)	**48**	Survival	0.059 (0.02)	0.19 (0.03)	1.85 (0.51)	LL.3
	96	Survival	0.024 (0.01)	0.05 (0.01)	2.99 (1.85)	LL.3
Macrobrachium amazonicum (µg/L)	**48**	Survival	0.17 (0.06)	0.63 (0.09)	1.72 (0.37)	LL.3
	96	Survival	0.04 (0.01)	0.10 (0.01)	2.30 (0.38)	LL.3
Danio rerio (mg/L)	**72**	Hatching	0.571 (0.226)	3.210 (0.588)	1.27 (0.26)	LL.3
	144	Mortality	0.561 (0.142)	1.680 (0.689)	-1.09 (0.46)	W1.4
	144	Edema	0.236 (0.156)	0.254 (0.050)	-15.83 (179.53)	W1.3
	144	Tremors	0.094 (0.036)	1.490 (1.106)	-0.79 (0.20)	LL.3
	144	Side-ways[a]	0.135 (0.061)	0.218 (0.028)	-4.55 (3.30)	LL.3
Daphnia similis **(**µg/L)	**24**	Immobilization	2.65 (1.68)	3.97 (0.93)	-4.45 (5.79)	LL.3
	48	Immobilization	0.79 (0.14)	7.87 (1.16)	-0.66 (0.11)	W1.3

a – larvae positioned side-ways (extreme loss of equilibrium); LL.3 - log-logistic 3 parameters function; W1.3 - Weibul 3 parameters function; W1.4 - Weibul 4 parameters function

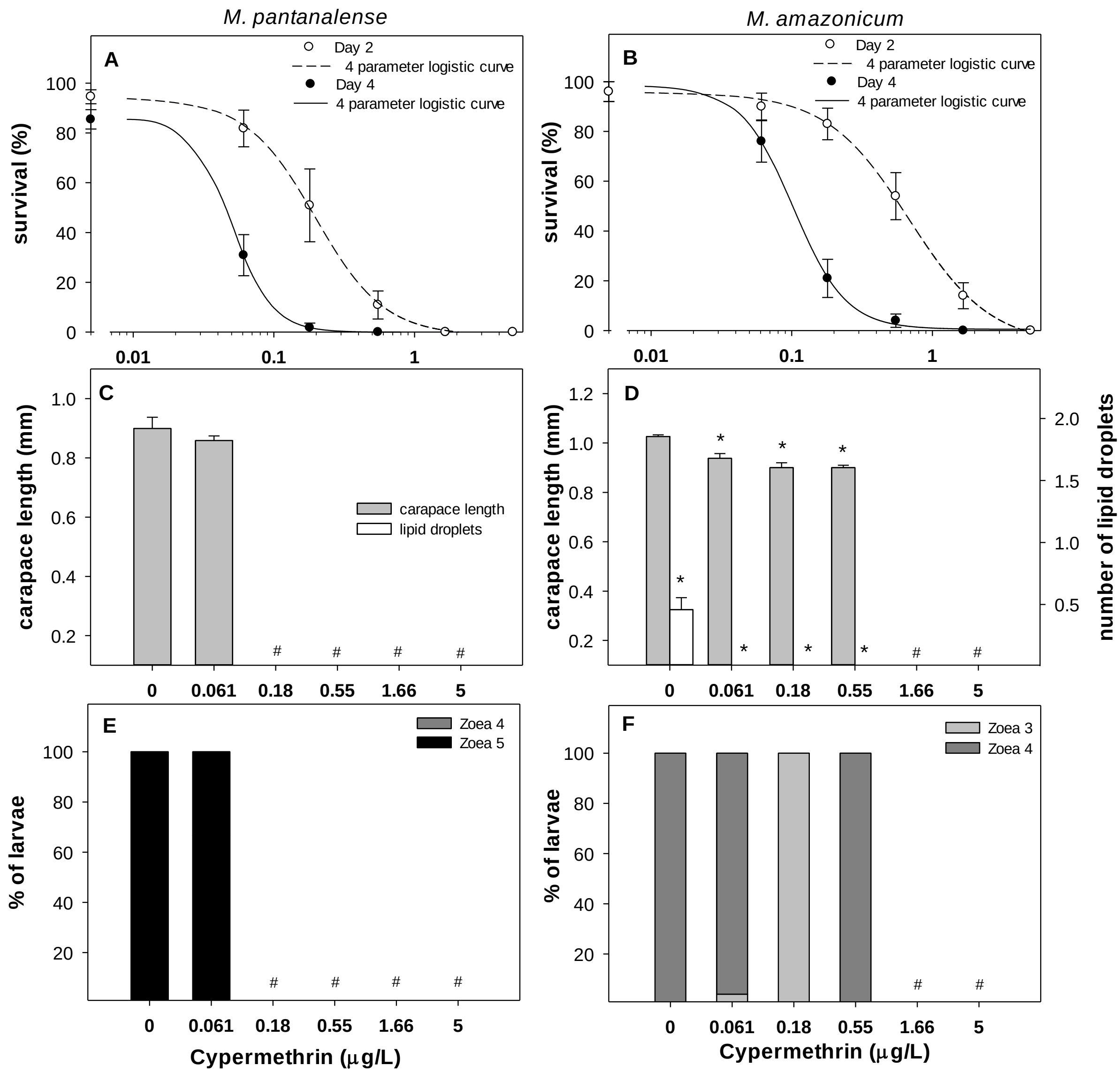

Figure 4 – Cypermethrin effects (through the formulation Barrage®) in larvae of *Macrobrachium pantanalense* and *Macrobrachium amazonicum* after 4 d of exposure: survival (A and B) – control is represented on the Y axis; carapace length (C and D); developmental stage (E and F). Asterisks denote statistically significant differences relative to the control ($p<0.05$). Values represent means and the error bars represent standard errors.

The development of larvae was delayed by CYP (**Fig. 4 E and F**). Concentrations as low as 0.061 µg/L (*M. pantanalense*) and 0.18 µg/L (*M. amazonicum*) caused some larvae to stay in zoea IV and III, respectively. The developmental stages and the CYP concentrations are significantly associated for *M. pantanalense* (χ^2=97.77, df=1, p<0.001) and *M. amazonicum* (χ^2=384.63, df=3, p<0.001).

3.2.2 Fish Embryo Toxicity Test (FET)

CYP caused significant effects on survival, hatching success and occurrence of deformities in zebrafish embryos (**Fig. 5**). The LC_{10} and LC_{50} values are shown in **Table 2**. Zebrafish were less sensitive than the shrimp larvae, with the LC_{50} differing in about two orders of magnitude.

Mortality of zebrafish exposed to CYP followed a dose response curve, increasing with concentration and with the duration of exposure (**Fig. 5 A**).

Hatching was stimulated for intermediate concentrations of CYP. At day 2, 76.7% of the eggs exposed to concentration 250 µg/L hatched, whereas only 8.1% of the eggs under control conditions hatched (**Fig. 5B**). Moreover, high concentrations of CYP (namely 4000 and 10000 µg/L) delayed hatching and decreased the hatching success.

Increasing concentrations of CYP also augmented the occurrence of tail malformation ($F_{8,19}$=18.068, p<0.001) and pericardial edema ($F_{8,19}$=47.240, p<0.001). Significant effects at day 6 were observed for concentrations above 250 µg/L (**Fig. 5C and 5E**). Moreover, CYP caused lost of equilibrium (**Fig. 5D**). For low concentrations a slight loss of equilibrium was observed, with larvae showing signs of unbalance; for concentrations equal and above 250 µg/L most of the larvae were side-laying. Larvae exposed to concentrations equal and above 250 µg/L also showed tremors (H=25.814, df=8, p=0.001). At day 6, such effect follows a dose response curve (**Fig. 5F**).

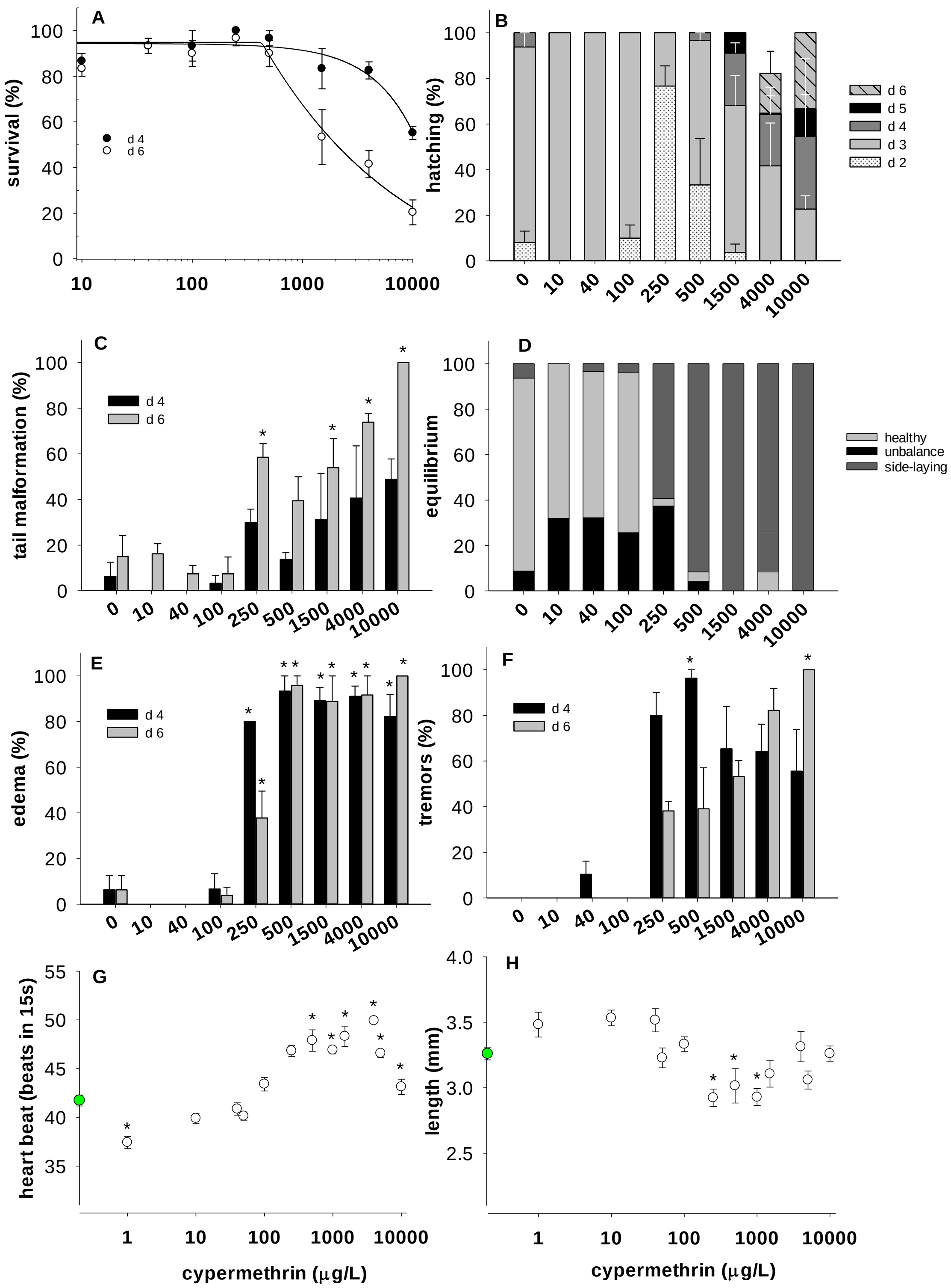

Figure 5 – Cypermethrin effects in zebrafish embryos: survival at day 4 and 6 (A); hatching per day as a percentage of living larvae: day 2, 3 4 5 and 6 (B); tail

malformations at day 6 (C); equilibrium at day 6: healthy, unbalance and sideways (D); heartbeat measured at 48h for 15 seconds (E); larvae length at day 6 (F). Graphs G and H represent pooled data from two independent tests; controls are represented on the Y axis. The asterisks (*) indicate significant differences compared to the control ($p<0.05$). Values represent means and the error bars represent standard errors.

Concerning the heartbeats and length, results did not follow a dose response curve. Heartbeat was affected by CYP ($F_{12,43}=21.668$, $p<0.001$), being lower than control for larvae exposed to 1 µg/L, and higher than control for larvae exposed to concentrations equal and above 500 µg/L (**Fig. 5G**). Larvae length was also affected by CYP (H=83.385, df=12, $p<0.001$), with larvae exposed to intermediate concentrations (250, 500 and 1000 µg/L) showing reduced length (**Fig. 5H**).

3.2.3 Immobilization test with *D. similis*

CYP increased the immobilization of daphnids after 24 and 48 h of exposure, following a dose-response curve (**Fig. 6**). The 48h-EC_{50} was 51.22 µg/l (**Table 2**).

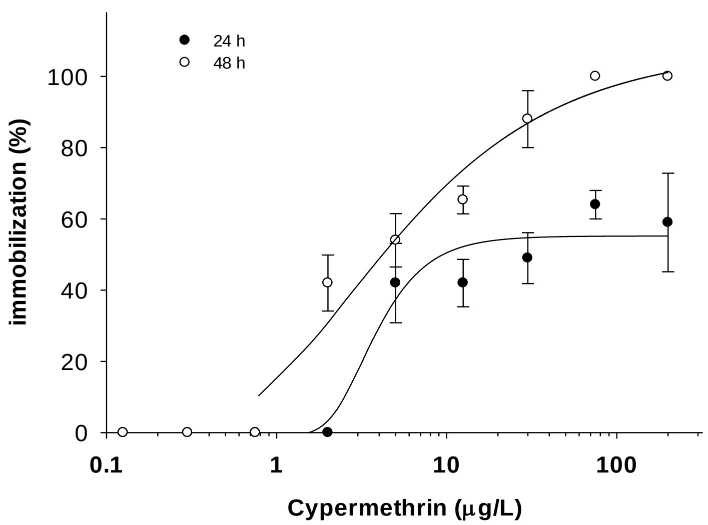

Figure 6 – Immobilization of neonates of *Daphnia similis* after 24 and 48 h of exposure to CYP (symbols represent means and the error bars represent the standard error)

4. Discussion

In this work we intended to assess if the toxicity of CYP, a widely used pesticide in Pantanal, is better achieved by toxicity tests with an endemic species - *M. pantanalense* – than standard species. Toxicity of copper was also tested since this is a standard compound and would be useful to assess the suitability of *M. pantanalense* for toxicity tests (as no previous literature reports their use). Moreover, both CYP and Cu-based pesticides are currently used worldwide (Dobhal et al., 2014).

Both Cu and CYP are toxic to the test species, affecting their survival, development and occurrence of deformities. For Cu the species sensitivity decreased in the following order: *M. pantanalense* > *D. similis* > *D. rerio* > *M. amazonicum*. The variability in the sensitivity is very high, with the 96h-LC_{50} for *M. pantanalense* being 3 orders of magnitude lower than the 120h-LC_{50} for *M. amazonicum*. For comparison of the results of the present study and results of previous studies, **Table 3** shows the LC_{50} values for freshwater shrimp, zebrafish and *Daphnia* exposed to Cu.

M. amazonicum is one of the least sensitive species among shrimps and also the least sensitive among the test species in this study (**Table 3**). *M. pantanalense* showed reduced carapace length, similarly to larvae of *Penaeus monodon* (Chen and Lin, 2001) for Cu concentrations 0.07 mg/L and above. The reduced carapace length of *M. pantanalense* agrees with the delay in the larval developmental, indicating decreased growth and molting frequency, which might be a consequence of reduced feeding, i.e, reduced energy uptake. This hypothesis is supported by a previous study that reported reduced feeding of *P. monodon* juveniles exposed to Cu (Chen and Lin, 2001).

Table 3 - LC_{50} values for shrimp, *Daphnia* and zebrafish exposed to Cu

Species	LC_{50} (µg/L)	Duration (h)	Reference
Shrimp			
Litopenaeus vannamei	39150	48	(Frías-Espericueta et al., 2008)
Macrobrachium amazonicum	2880	120	**this study**
Macrobrachium dayanum	135	72	(Lodhi et al., 2006)
Macrobrachium hendersodayanum	445	96	(Patil and Kaliwal 1986 cit. in Lodhi et al., 2006)
Macrobrachium kistenensis	71	48	(Ghate and Mulherkar 1979 cit. in Lodhi et al., 2006)
Macrobrachium lamarrei	87	72	(Lodhi et al., 2006)
Macrobrachium lamarrei	63	96	(Murti and Shukla 1984 cit in Lodhi et al., 2006)
Macrobrachium pantanalense	3.24	96	**this study**
Macrobrachium rosenbergii	1120	48	(Sánchez and Delgado, 2006)
Paratya australiensis	20	96	(Daly et al., 1990)
Penaeus japonicus	3-50	48	(Bambang et al., 1995)
Zebrafish			

	37	96	(de Oliveira-Filho et al., 2004)
	700	120	(Hua et al., 2014)
	1440	144	**this study**
	1870	96	**this study**
Daphnia			
Daphnia magna	57 (H=180)	48	(Naddy et al., 2002)
Daphnia magna	43 (H=180)	48	(Naddy et al., 2002)
Daphnia magna	119 (H=179)	48	(Barata et al., 1998) (a)
Daphnia magna	23 (H=179)	48	(Barata et al., 1998) (a)
Daphnia similis	11 (H=45)	48	(Arauco et al., 2005) (b)
Daphnia similis	5 (H=40-48)	48	(de Oliveira-Filho et al., 2004)
Daphnia similis	51.2 (H=175)	48	**this study**

H: hardness, expressed as mg $CaCO_3$/L

Concerning zebrafish, the toxicity value found in this study is higher than literature values, which can be due to several factors, namely the genetic variation in zebrafish lines and the characteristics of the test water (namely hardness). However, results concerning survival and hatching are concordant with those of a previous study which reported effects on survival for Cu concentrations equal and above 0.25 mg/L and delayed hatching for concentrations equal and above 0.5 µg/L (Hua et al., 2014). Zebrafish also showed reduced length and heartbeat, as well as increased incidence of pericardial edema, which is concordant with the findings of previous works (Bai et al., 2010; Johnson et al., 2007). Moreover, the fact that the mortality occurred mainly in the first 24 h of exposure has been reported previously for Cu (Johnson et al., 2007) and other metals (Chow and Cheng, 2003).

Regarding *Daphnia*, the EC_{50} for *D. similis* is within the range found for other *Daphnia* species at the same hardness. Hardness has a protective role in the toxicity of metals to aquatic organisms – increasing hardness reduces Cu toxicity, i.e., increases the EC_{50} value, which is due to competition between Cu and Ca ions for uptake (Ryan et al., 2009). Thus, it was expected that the EC_{50} for *D. similis*, determined at a hardness level of 175 mg $CaCO_3$/L, was higher than those reported in previous studies for the same species tested at lower hardness levels, concordantly with values in **Table 3**.

For CYP the species sensitivity decreased in the following order: *M. pantanalense* > *M. amazonicum* > *D. similis* > *D. rerio*. As for Cu, the variability in the sensitivity is very high, with the 96h-LC_{50} for *M. pantanalense* being 4 orders of magnitude lower than the 144h-LC_{50} for zebrafish. High toxicity of CYP to crustaceans is not surprising since this compound, as other pyrethroids, is designed to control arthropod pests. For comparison of the toxicity values with those reported in previous studies, **Table 4** shows the LC_{50} values for shrimp, *Daphnia* and zebrafish exposed to CYP. Note, however, that the results of the present study were obtained with a commercial formulation containing CYP (Barrage®) and not with pure CYP.

The LC_{50} values found for *M. amazonicum* and *M. pantanalense* are in the same range of those found for other shrimp species, being *M. amazonicum* however the least sensitive. Other effects of CYP to both shrimp species included reduced growth and delayed development. These effects were also observed in the shrimp *Palaemonetes argentinus* exposed to CYP concentrations as low as 0.0001 µg/L (Collins and Cappello, 2006).

Concerning zebrafish, the 144h-LC_{50} value is much higher than values reported in previous studies. Such difference might be related to the experimental conditions. For instance, the low value obtained by Sathya et al. (2014) is due to the daily renewal of the test medium, thus, counteracting the trend for the decrease of the CYP concentration in the medium due to degradation. In addition, the differences in the toxicity values between this and other studies might be related to the fact that a formulation was used. Formulations contain not only the active ingredient (CYP in this case), but also "inert ingredients", constituting a mixture. As a result, the toxicity of formulations is often under- or overestimated compared to that of the active ingredient (Pereira et al., 2009). Our results suggest that the toxicity of the CYP formulation commercialized as Barrage® is lower than the toxicity of pure CYP, particularly for zebrafish and *Daphnia*. Moreover, CYP undergoes a rapid degradation, as shown by the chemical analysis (**Table S2**, Supplementary Material).

Table 4 - LC_{50} values for shrimp, zebrafish and *Daphnia* exposed to cypermethrin

Species	**LC_{50} (µg/L)**	**Duration (h)**	**Reference**
Shrimp			
Macrobrachium amazonicum	0.10	96	**this study**
Macrobrachium pantanalense	0.05	96	**this study**
Macrobrachium rosenbergii	0.031	96	(Pillai et al., 1989 cit. in Collins; Cappello, 2006)
Palaemonetes argentinus	0.0020	96	(Collins and Cappello, 2006)

Paratya australiensis	0.019	96	(Kumar et al., 2010)
Zebrafish			
	0.05	96	(K.Sathya et al., 2014)
	73.0	144	(Yang et al., 2014)
	65	144	(DeMicco et al., 2010)
	1680	144	**this study**
Daphnia			
Daphnia magna	0.26	48	(Keith and Walker, 1992)
Daphnia magna	0.00061	96	(Kim et al., 2008)
Daphnia similis	7.87	48	**this study**

CYP caused several effects in zebrafish. At 250 µg/L and above, larvae exhibited tail malformation (curvature), pericardial edema and tremors. However, mortality occurred only for concentrations 1500 µg/L and above. In general, these effects, which followed a dose-response curve, were also reported in previous studies with the same species (DeMicco et al., 2010; Sathya et al., 2014; Shi et al., 2011; Xu et al., 2010; Yang et al., 2014). Nevertheless, none of these studies reported early hatching of embryos, which suggests that this effect is not caused by CYP but probably by other ingredients of the formulation. Such an effect is not commonly reported in the literature. Early hatching of zebrafish embryos was reported after exposure to a wide range of compounds including the pyrethroid flumethrin (Carlsson et al., 2013), and the solvents DMSO and acetone (Hallare et al., 2006). Indeed, exposure to the pyrethroid flumethrin caused not only early hatching, but also increased heart rates, tremors and unbalance (side-laying), similarly to the Barrage® formulation. This, allied to the fact that pesticides formulations may contain a mixture of chemicals (Elhalwagy and Zaki, 2009) raises the hypothesis that the toxicity of Barrage® to zebrafish may be due to the presence of other pesticides in the formulation. On the other hand, we could hypothesize that the observed effects, namely early hatching, could be due to the solvents: C8, C9 and C10 aromatics. Actually, many formulations of pyrethroid insecticides are based in a hydrocarbon mixture (Magdalan et al., 2009). There is scarce information on the toxicity of this mixture to fish. In the environment, early hatching leads to early exposure of the larvae to the water, outside the chorion and, thus, without this protective barrier. As a consequence, fish larvae could be more exposed to chemicals which could result in more adverse effects. Indeed, the early hatching of zebrafish exposed to 250 µg CYP/L might explain the increased occurrence of tail malformation, which is higher for zebrafish exposed to 250 µg CYP/L than 500 µg CYP/L. The same hypothesis is applied to the length, which is more affected for fish exposed to 250 µg CYP/L than 500 µg CYP/L.

The toxicity value found for *D. similis* is much higher than toxicity values reported in previous studies for other *Daphnia* species, which can be due to differences in species sensitivity or, as mentioned previously, to the fact that a formulation, not only the active ingredient, was used. Previous studies showed that *D. magna* is very sensitive to this insecticide with concentrations as low as 0.0002 ng/L causing a significant delay in the time required for production of the first brood (Kim et al., 2008).

The results of the present study raise concern about the effects of CYP to the aquatic biota in Pantanal. As mentioned previously, the formulation Barrage® is widely used in this region for control of a wide variety of insects, including for domestic use.

This compound is very toxic for the endemic shrimp *M. pantanalense*, as well as for the other crustacean species (*M. macrobrachium* and *D. similis*). Moreover, CYP is an endocrine-disruptor pesticide (Feng et al., 2015) and can affect several aspects of fish reproduction. For instance low concentrations (< 0.004 µg/L) diminished the response of male fish (*Salmo salar*) to pheromones released by the females, as well as decreased eggs fertility (Moore and Waring, 2001). In addition, CYP is also able to induce apoptosis and immunotoxicity in zebrafish (Jin et al., 2011). These factors suggest an important effect in aquatic organisms. Furthermore, despite the scarce information on the environmental concentrations in the surface water, CYP concentrations between 0.02 and 0.52 µg/L were detected in rainwater (Moreira et al., 2012). The maximum value is about 10 fold higher than the 96h-LC_{50} for *M. pantanalense* and 5 times the 96h-LC_{50} for *M. amazonicum*. However, conclusions about the risk for Pantanal aquatic species, namely shrimps, cannot be withdrawn due to scarce information on environmental concentrations and on the amount sold locally. In opposition, it is important to highlight the fast degradation of CYP which, in natural ecosystems is accelerated by biodegradation and adsorption to the soils (Liu et al., 2004). Both these factors contribute to a decrease of CYP toxicity.

The high sensitivity of *M. pantanalense* to both copper and CYP, compared to the tested species, highlights the importance of using endemic species in risk evaluations. For a better comprehension of the ecological risk in the Pantanal region we suggest that further studies should be carried out with endemic species in this region, representative of different levels in the trophic chain. Moreover, this study emphasized the importance of testing pesticides toxicity as commercial formulations, as some of the effects of formulations might not be due to the active ingredient, but to the inert ingredients or their interaction. Furthermore, our results suggest that the endemic shrimp species *M. pantanalense* can be successfully used as a test species in ecotoxicology.

5. Conclusion

The Pantanal endemic shrimp species *M. pantanalense* showed to be more sensitive to the two studied compounds than the other species tested. This suggests that when assessing environmental impact of sensitive biomes such as Pantanal, risk may be underestimated by using standard species usually used in ecotoxicology. A better assessment can be achieved by using endemic species representative of different trophic levels. The wide use of the CYP formulation (Barrage®) in Pantanal, allied to its high toxicity (96 h-LC_{50} for *M. pantanalense* as low as 50 ng/L) and to the hydrological regimes of the Pantanal flood plains, together with the lack of knowledge of environmental concentrations raise concern about the effects of this compound to the aquatic organisms of this fragile biome. Monitoring studies and a better characterization of risk must be conducted to avoid deleterious effects on aquatic communities.

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CAPÍTULO 3 – CONSIDERAÇÕES FINAIS

Esse estudo apresentou dados referentes a sensibilidade avaliada por meio da exposição aguda de *Macrobrachium pantanalense* ao cobre (Cu), um composto de referência e cipermetrina um pesticida amplamente utilizado na região do Pantanal, por meio da formulação Barrage®, em comparação com outras espécies aquáticas.

A alta sensibilidade do *M. pantanalense* ao Cu e CYP, em comparação com as espécies testadas, destaca a importância da utilização de espécies endêmicas nas avaliações de risco. Sendo assim, nossos resultados sugerem que as espécies de camarões endêmicos *M. pantanalense* podem ser utilizados com sucesso como uma espécie de teste em ecotoxicologia.

Além disso, a elevada utilização de cipermetrina no Pantanal, aliada à sua toxicidade e ao regime hídrico desta região, a par da ausência de conhecimento das concentrações ambientais deste composto levantam preocupação acerca dos efeitos deste composto para os organismos aquáticos deste frágil bioma.

Este estudo também enfatiza a importância de pesquisas futuras relacionadas a toxicidade dos pesticidas ser também avaliada sob a forma de formulações comerciais, visto que a toxicidade da formulação difere da cipermetrina pura.

Para uma melhor compreensão do risco ecológico na região do Pantanal, sugerimos que novos estudos devem ser realizados com espécies endêmicas da região, representativas de diferentes níveis na cadeia trófica. Assim como, estudos de monitoramento e uma melhor caracterização do risco devem ser realizados para evitar os efeitos deletérios sobre as comunidades aquáticas.

SUPPLEMENTARY MATERIAL

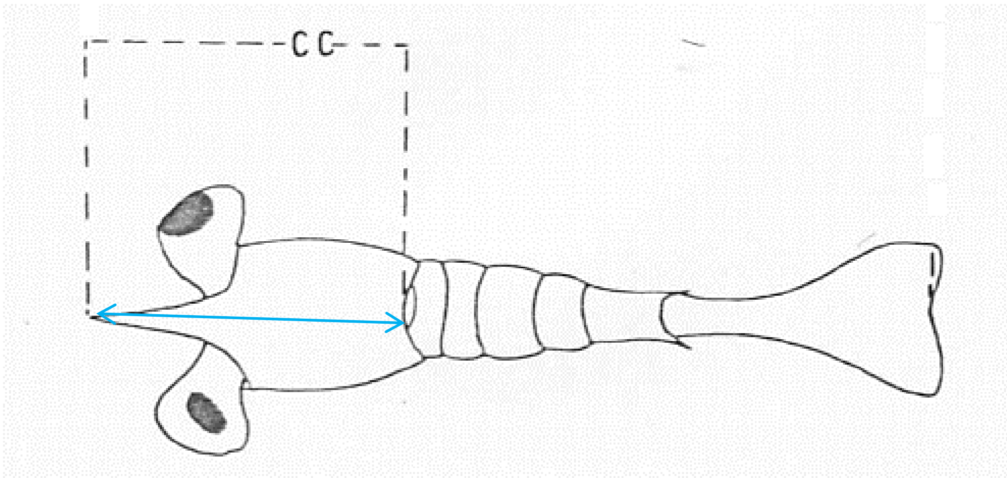

Fig. S1- The length CC represents the carapace length. It is measured over the distance between the end of the rostrum (tip) to the median posterior edge of the carapace – represented as a blue line (adapted from Vega-Perez, 1984).

Table S1 – Results of the chemical analysis of copper

test species	time (h)	nominal concentration (µg/L)	measured concentration (µg/L)	recovery (%)
Danio rerio	0	125	100	80
		250	196	78
		500	421	84
		1000	835	84
		2000	1909	95
		4000	3702	93
	144	125	65.7	53
		250	157	63
		500	264	53
		1000	630	63
		2000	799	40
		4000	1028	26
Daphnia similis	0	0.5	BDL	---
		1.3	BDL	---
		5	BDL	---
		10	15.3	153
		20	16.8	84
		50	42.5	85
		80	71.8	90
		120	102	85
		250	215	86
	48	0.5	BDL	---
		1.3	BDL	---
		5	BDL	---
		10	BDL	---
		20	13.8	69
		50	37.2	74
		80	65.2	82
		120	92.5	77
		250	199	80

BDL – below detection limit

Table S2 – Results of the chemical analysis of cypermethrin

test species	time (h)	nominal concentration (µg/L)	measured concentration (µg/L)	recovery (%)
Danio rerio	0	10	9.6	96
		40	39	98
		100	95	95
		250	240	96
		500	420	84
		1500	1400	93
		4000	3500	88
		10000	9000	90
	144	10	0.54	5
		40	0.82	2
		100	2.4	2
		250	6.4	3
		500	10	2
		1500	55	4
		4000	230	6
		10000	730	7
Daphnia similis	0	0.02	0.018	90
		0.13	0.15	115
		0.75	0.82	109
		5	5.8	116
		30	34	113
		200	210	105
	48	0.02	<10 ng/L	---
		0.13	0.034	26
		0.75	0.076	10
		5	1.8	36
		30	7.1	24
		200	160	80

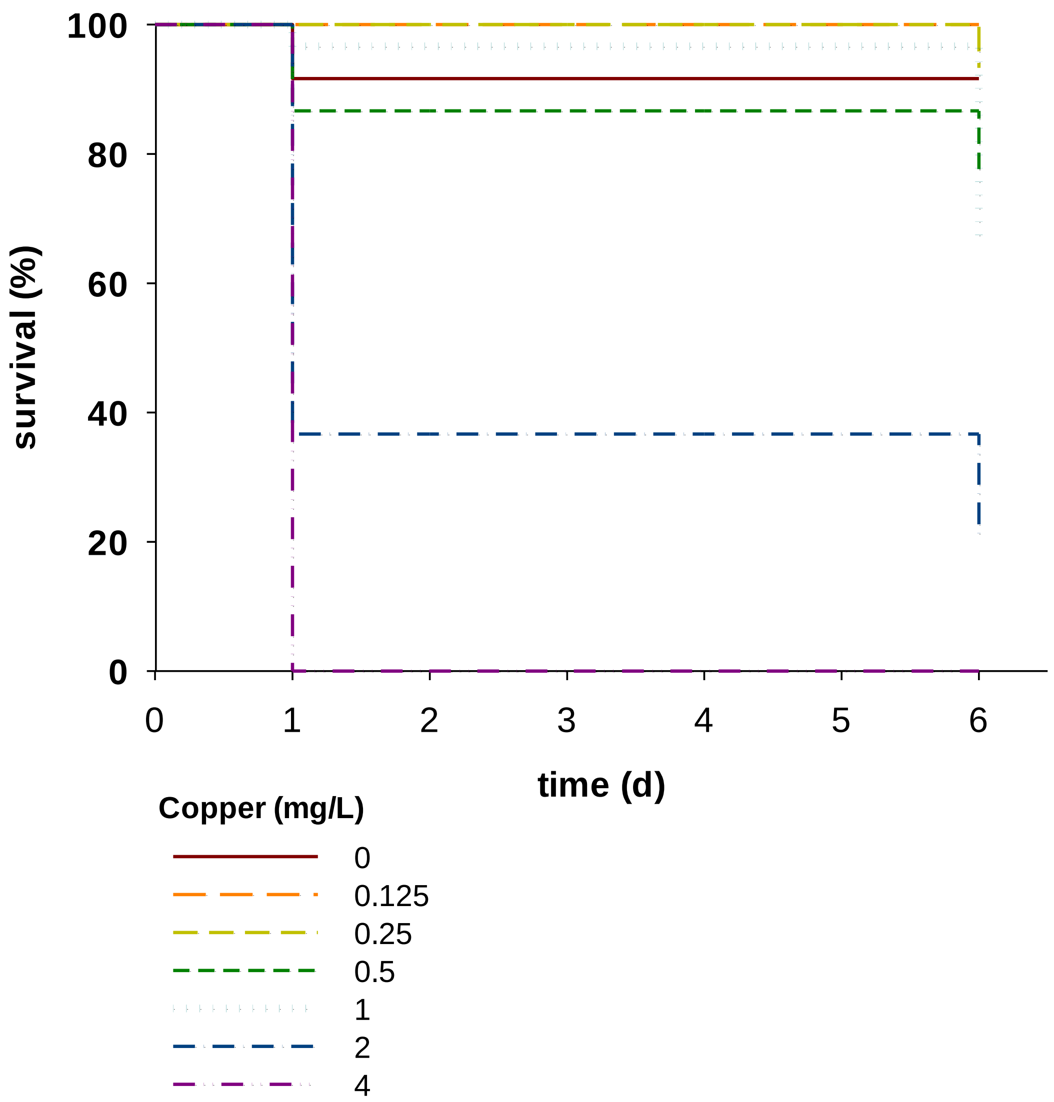

Fig. S2 – Survival of *Danio rerio* embryos exposed to Cu during 6 days (144h)