UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

ANTONIO JOSÉ GAZONATO NETO

AVALIAÇÃO DA TOXICIDADE DE METAIS E PESTICIDAS, ISOLADOS E EM MISTURA, SOBRE DUAS ESPÉCIES DE OLIGOCHAETA NATIVOS NEOTROPICAIS.

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Tese apresentada ao Programa de Pós-graduação em Ecologia e Recursos Naturais do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Ciências, área de concentração em Ecologia e Recursos Naturais.

Orientadora: Profa. Dra. Odete Rocha

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UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ecologia e Recursos Naturais

Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado do candidato Antônio José Gazonato Neto, realizada em 13/03/2018:

Profa. Dra. Odete Rocha
UFSCar

Profa. Dra. Maria da Graça Gama Melão
UFSCar

Profa. Dra. Ana Teresa Lombardi
UFSCar

Profa. Dra. Eny Maria Vieira
USP

Profa. Dra. Adrislaine da Silva Mansano Dornfeld
USP

À minha avó Halum ॐ:

Um porto aonde sempre poderei voltar em tempos de tormenta.

“O deus que habita em mim saúda o deus que habita em você.”

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Ilusões de Vida

"Quem passou pela vida em branca nuvem
E em plácido repouso adormeceu;
Quem não sentiu o frio da desgraça,
Quem passou pela vida e não sofreu,
Foi espectro de homem - não foi homem,
Só passou pela vida - não viveu."

Francisco Otaviano

RESUMO

Dada a urgência em se utilizar espécies nativas como organismos-teste em estudos de toxicidade que reflitam as propriedades típicas dos sistemas tropicais e a necessidade de se analisar agentes tóxicos ou contaminantes não apenas de maneira isolada, mas em misturas que retratem as condições encontradas nos ecossistemas, este estudo teve por objetivo testar a adequação de duas espécies nativas de invertebrados como organismos-teste na determinação da toxicidade de metais e pesticidas. Para isso foram realizados ensaios de toxicidade aguda e crônica com as espécies nativas *Dero furcatus* e *Allonais inaequalis* (Oligochaeta, Naididae) sob exposição de curto e longo prazo a metais e pesticidas de ampla frequência de ocorrência em sistemas aquáticos brasileiros. Dentre os metais testados, cádmio, mercúrio e cobre se destacam por sua alta toxicidade e amplo uso em processos industriais, bem como por seu alto poder de bioacumulação ao longo da cadeia trófica, e mesmo metais como o manganês, embora com baixa toxicidade, podem causar danos a longo prazo sobre a biota aquática, principalmente em misturas com potencial sinergismo. Foram testados os pesticidas carbofurano e diuron, compostos de ampla utilização nos sistemas agrícolas brasileiros. A 96h CL_{50} do cloreto de cádmio foi 627 µg L^{-1} e 364 µg L^{-1} para *A. inaequalis* e *D. furcatus*, respectivamente. Para o cloreto de mercúrio, a 96h CL_{50} foi 129 µg L^{-1} para *A. inaequalis* e de 92 µg L^{-1} para *D. furcatus*. A 96h CL_{50} para o sulfato de cobre foi 25,75 µg L^{-1} para *A. inaequalis* e 31,39 µg L^{-1} para *D. furcatus.* Para o sulfato de manganês, a 96h CL_{50} foi 28.155,16 µg L^{-1} para *A. inaequalis* e 18.250,62 µg L^{-1} para *D. furcatu*s. A sensibilidade das espécies nativas de oligoquetos foi superior ou similar à de espécies de anelídeos utilizados mundialmente como organismos-teste, tais como *Tubifex tubifex* e *Lumbriculus variegatus.* Isto demonstra a vantagem de se utilizar espécies nativas tropicais em ensaios de toxicidade. Os testes de toxicidade crônica mostraram que mesmo sob concentrações sub-letais muito baixas e ambientalmente relevantes, os metais e pesticidas selecionados afetaram negativamente importantes aspectos do ciclo de vida dos oligoquetos, como o crescimento e a sobrevivência, o que a longo prazo poderia acarretar em uma redução na população natural e consequente extinção local. As misturas de metais revelaram sinergismo, implicando na probabilidade de potencialização de efeitos tóxicos quando os metais ocorrem juntos no ambiente, demonstrando por exemplo, que embora isoladamente o manganês não apresente elevada toxicidade, sua ocorrência natural concomitante e a interação com outros metais pode aumentar os efeitos tóxicos para níveis maiores que a simples adição dos seus efeitos isolados.
Palavras-chave: Espécies nativas; Bentos; Metais tóxicos; Misturas; Pesticidas.

ABSTRACT

It is recognized the need of using native species as test organisms in toxicity studies in order to adequately reflect the typical properties of tropical systems and impacts as those of toxic or contaminating agents acting not only in isolation, but also in mixtures that depict the conditions found in ecosystems, this study aimed to test the suitability of two native species of invertebrates as test organisms in the determination of metals and pesticides toxicity. Acute and chronic toxicity tests were performed with the native species *Dero furcatus* and *Allonais inaequalis* (Oligochaeta, Naididae) under short and long term exposure to metals and pesticides with high frequency of occurrence in Brazilian aquatic systems. Among tested metals, cadmium, mercury and copper stand out for their high toxicity and wide use in industrial processes, as well as their high bioaccumulation power along the trophic chain. Even metals such as manganese, although with low toxicity, can cause long-term damage on aquatic biota, especially in mixtures with potential synergism. The pesticides carbofuran and diuron, compounds, widely used in Brazilian agricultural systems, were tested. The 96h LC_{50} of cadmium chloride was 627 µg L^{-1} and 364 µg L^{-1} for *A. inaequalis* and *D. furcatus*, respectively. For mercury chloride, 96h LC_{50} was 129 µg L^{-1} for *A. inaequalis* and 92 µg L^{-1} for *D. furcatus*. 96h LC_{50} for copper sulfate was 25.75 µg L^{-1} for *A. inaequalis* and 31.39 µg L^{-1} for *D. furcatus*. For manganese sulfate, 96h LC_{50} was 28155.16 µg L^{-1} for *A. inaequalis* and 18250.62 µg L^{-1} for *D. furcatus*. The sensitivity of the native species of oligochaetes was superior or similar to that of annelid species used worldwide as test organisms, such as *Tubifex tubifex* and *Lumbriculus variegatus*. This demonstrates the advantage of using native tropical species in toxicity assays. Chronic toxicity tests showed that even under very low and environmentally relevant sublethal concentrations, selected metals and pesticides negatively affected important aspects of the life cycle of oligochaetes, such as growth and survival, which in the long run could lead to a reduction in the natural population and consequent local extinction. Mixtures of metals have shown synergism, implying the probability of potentiation of toxic effects when metals occur together in the environment, demonstrating for example that although manganese alone does not present high toxicity, its concomitant natural occurrence and interaction with other metals may increase the toxic effects to greater levels than the simple addition of their isolated effects.

Keywords: Native species; Benthos; Toxic metals; Mixtures; Pesticides.

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Sumário

Estruturação da tese

O item "Resultados e Discussão" do presente estudo foi escrito na forma de manuscritos em inglês para publicação, compostos de Resumo, Introdução, Materiais e Métodos, Resultados, Discussão e Referências, e formatados de acordo com as normas do periódico internacional Ecotoxicology and Environmental Safety. Desse modo, no primeiro capítulo da tese são introduzidos os aspectos gerais da pesquisa realizada em relação aos compostos metálicos e pesticidas utilizados, bem como sobre a importância do estudo de misturas, de testes crônicos e do uso de organismos nativos nos testes de toxicidade. A este capítulo seguem os objetivos e hipóteses de trabalho, respondidos no decorrer dos manuscritos apresentados. Por fim, as conclusões e considerações gerais sobre o trabalho e síntese dos resultados e conclusões atingidas com as pesquisas desenvolvidas.

1. Introdução e Justificativa

1.1 Precedentes dos testes de toxicidade

A urbanização e o advento das superpopulações humanas trouxeram à tona a urgência de se avaliar a saúde dos sistemas ecológicos assim como de seus componentes bióticos, dentre os quais nos inserimos (Carvalho, 2017). Estas avaliações são usualmente feitas por meio de medidas de variáveis ecológico-sanitárias que permitem mensurar a qualidade de um ambiente, envolvendo por exemplo, estudos microbiológicos, físicos, químicos e radioativos, que refletem a degradação natural ou antropogênica e que possibilitam mensurar, atenuar e, em alguns casos remediar os riscos ecológicos aos ecossistemas atingidos pelos contaminantes (Andrade et al., 2010; Kuperman et al., 2004). Dentre estas ferramentas destacam-se as análises ecotoxicológicas.

A rápida industrialização observada em países em desenvolvimento, tais como o Brasil, somada à pobreza, poluição e uma precária infra-estrutura urbana acentuam ainda mais o problema: a maioria de suas cidades apresenta tratamento incompleto de esgoto, e, por este motivo, despejam poluentes residuais em rios, lagos e canais (Grover and Kaur, 1999).

Além de seu papel inquestionável na preservação da vida, os corpos hídricos são objeto de grande preocupação nos últimos tempos devido às gravíssimas crises hídricas que têm assolado países ao redor de todo globo, inclusive o Brasil, país que contempla em seu território aproximadamente 10% da água doce do Planeta, mas que, contrariando seu potencial natural, tem enfrentado racionamentos de água e, por vezes, recorrido ao volume morto de seus reservatórios para o abastecimento da população (Andrade et al., 2010; Corbi et al., 2006), apesar de geralmente conterem maior concentração de contaminantes.

Soma-se à questão da disponibilidade hídrica a evidente problemática da qualidade hídrica, resultado direto da ação antrópica de contaminação dos mesmos por meio de resíduos urbanos (industriais e domésticos), tais como o despejo de efluentes não tratados bem como de resíduos advindos da agricultura, os quais geram um quadro de crise sócio-ambiental, uma vez que a saúde do meio que nos circunda afeta diretamente a saúde das populações humanas e de toda a biota contemplada nestes ecossistemas, prejuízos de responsabilidade governamental, mas que também recaem sobre os ombros da esfera civil (Corbi et al., 2006; Silva et al., 2007).

A poluição dos recursos hídricos por compostos químicos tóxicos é um grave e crescente problema em todo o planeta e que, apesar de ser um tópico com legislação específica em diversos países, continua a ocorrer pela falta de controle e fiscalização sobre o descarte de efluentes domésticos, industriais e agrícolas, as principais fontes responsáveis pela contaminação de ambientes aquáticos (Kumar, 2012; Lemos et al., 2005; Matsumoto et al., 2006). Esta descarga deliberada ou a liberação acidental de substâncias químicas nocivas para o meio ambiente podem perturbar a estrutura e a função dos ecossistemas naturais e, portanto, representar uma grave ameaça à diversidade biológica (Kumar et al., 2012).

A poluição reduz a qualidade de vida em diversos aspectos, afetando nossa saúde e longevidade (Grover and Kaur, 1999). As substâncias poluentes podem ser responsáveis por efeitos deletérios nos organismos (Figura 1) tais como a alteração no funcionamento dos órgãos, no estado reprodutivo, na sobrevivência das espécies e alterações no tamanho das populações (Kumar, 2012). Além desses efeitos diretos à saúde, o perigo dos poluentes reside no fato de poderem ser mutagênicos ou tóxicos e levarem a diversos problemas como o câncer, arterosclerose, doenças cardiovasculares e envelhecimento precoce (Grover and Kaur, 1999).

Embora as análises químicas sejam o método primário através do qual os efluentes industriais são estudados em relação à toxicidade potencial, as limitações inerentes a tais abordagens são claras. Devido à natureza química complexa dos resíduos industriais, as análises químicas convencionais são limitadas em sua habilidade de caracterizar a composição destas misturas e permitir uma avaliação ecotóxica químico-específica. Em contraste às análises químicas, os bioensaios fornecem um meio de avaliar a toxicidade de misturas complexas sem um conhecimento prévio sobre a composição química das mesmas. A utilidade dos bioensaios resultou em seu uso na avaliação de uma vasta gama de efluentes e misturas industriais, uma demanda urgente quando lidamos com países com abundantes recursos hídricos, tais como o Brasil (Andrade et al., 2010; Claxton et al., 1998).

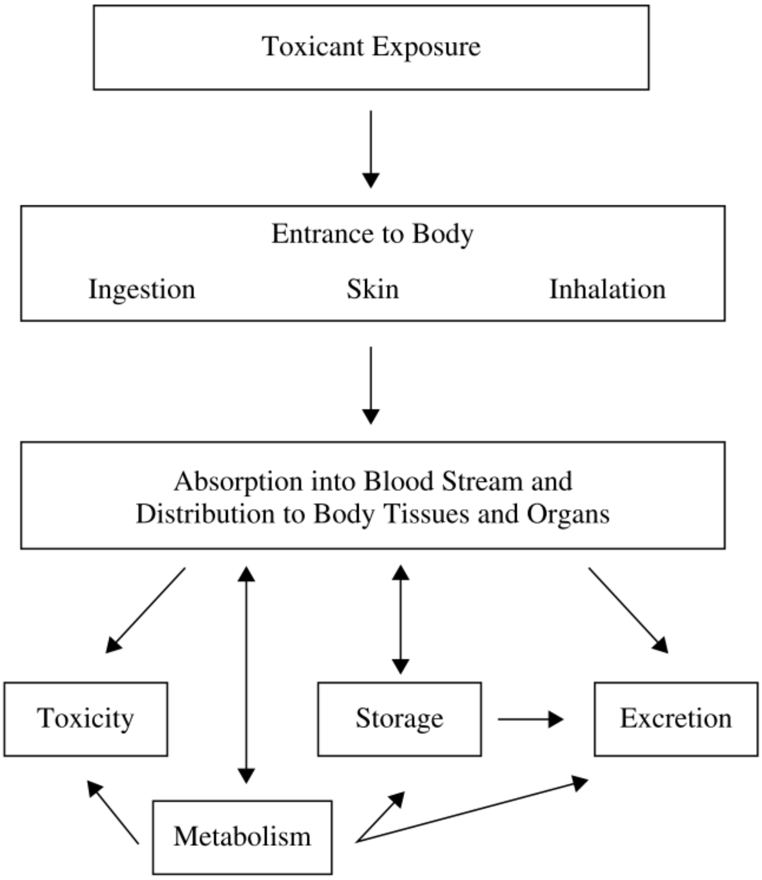

Figura 1. Os caminhos dos contaminantes nos vertebrados. Fonte: Hodgson, 2004.

1.2 A problemática dos metais

Apesar da inegável importância de inúmeros metais na manutenção de algumas funções básicas da vida, uma vez que são constituintes básicos de enzimas, hormônios e responsáveis pela manutenção dos processos metabólicos, bem como responsáveis pela prevenção de doenças (Andrade et al., 2010; CETESB, 2009; Sanders et al., 1998), altos níveis de compostos metálicos causam consequências nocivas e às vezes letais para a saúde humana, principalmente pelo seu alto potencial nefrotoxicante (Hodgson, 2004), devido à ligação que estes compostos inorgânicos tendem a formar com moléculas orgânicas, podendo desativar funcionalidades das mesmas (Sánchez-Bayo, 2012).

A presença de metais em sistemas aquáticos pode ser explicada, pelo menos parcialmente, por processos naturais típicos, como atividades vulcânicas ou erosão do solo (Parelho et al., 2014), todavia, os níveis elevados nas concentrações de metais são provavelmente o resultado direto da ação antrópica (Figura 2), principalmente por disposição de lixo industrial ou lixiviação do solo de áreas agrícolas (Kelepertzis, 2014;

Mansour and Sidky, 2002), especialmente no Brasil, principal consumidor de pesticidas desde o ano de 2008 (Albuquerque et al., 2016; Barbosa et al., 2015). O uso crescente de fertilizantes à base de metal em culturas de cana-de-açúcar, uma das principais monoculturas no Brasil (Azania et al., 2009), associado ao processo de desmatamento das florestas ripárias e a selagem das superfícies do solo, bem como o processo de erosão (Dellamatrice and Monteiro, 2014), culminaram em impactos significativos sobre os recursos hídricos circundantes, bem como nos ecossistemas adjacentes dentro da área de influência dessas culturas, uma vez que a maioria desses compostos metálicos são carreados do solo para os corpos d'água superficiais e subterrâneos pelo processo de lixiviação e, devido à alta solubilidade na água de alguns desses compostos, podem ser absorvidos por tecidos de plantas e animais, afetando assim organismos não-alvo em ecossistemas terrestres e aquáticos, assim como suas interfaces (Corbi et al., 2006; Ming-Ho Yu, 2004).

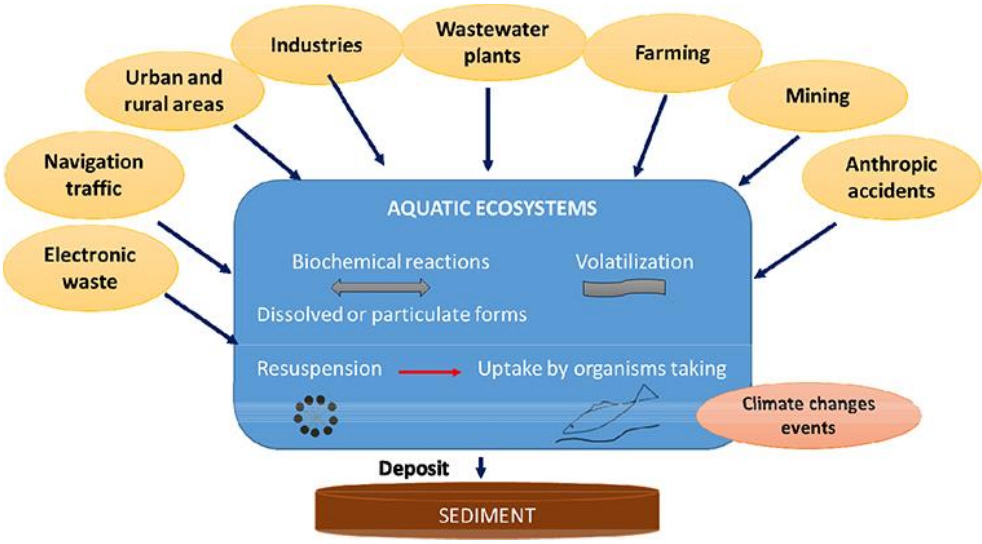

Figura 2. Principais formas de entrada dos metais nos ecossistemas aquáticos. Fonte: Gheorghe et al., 2017.

Além do uso inadequado do solo, o descarte inadequado de resíduos industriais e do esgoto sanitário também acarretam no aumento das concentrações de poluentes nos sistemas naturais, como os resíduos provenientes de metalúrgicas e indústrias de tintas, ambas responsáveis pela utilização de metais no decorrer da linha de produção e pelo descarte irresponsável dos subprodutos industriais, bem como os incineradores de lixo urbano e industrial, outra fonte de metais responsável pela volatização e pela subsequente formação de cinzas com altos teores de metais, majoritariamente mercúrio e arsênio (Andrade et al., 2010; Kuperman et al., 2004)

A mensuração dos impactos e distúrbios ambientais por meio do estudo e quantificação de metais não é uma prática recente; em 1986 Amiard relatou que flutuações naturais de metais em mexilhões se tornaram parâmetros indispensáveis para aferir os impactos da poluição local. Desse modo, entende-se que a biodisponibilidade de metais pesados nos ecossistemas não é causa imediata da degradação antropogênica, como a mineração e a queima de combustíveis fósseis, mas decorre inicialmente de fontes naturais, tais como o intemperismo físico e químico de rochas e solos, influenciado pelo clima, pela cobertura vegetal, pela topografia e pela geologia do solo (Andrade et al., 2010).

Seja como resultado da ação antrópica ou dos processos naturais, os metais são os poluentes mais comumente encontrados em meio aquático (Corbi et al., 2006) e, por este motivo, a contaminação por meio de metais tem ganhado destaque mundial, e se tornado, cada vez mais, objeto de publicações ao redor do mundo, uma vez que se enquadra como requisito fundamental nos estudos de qualidade dos recursos hídricos (Andrade et al., 2010; Mansour and Sidky, 2002).

Os níveis de exposição são determinantes quando caracteriza-se a toxicidade de um metal. O efeito dos poluentes metálicos, sejam eles metais isolados ou compostos que contenham metais em suas fórmulas, variam de acordo com os limites de tolerância à concentração ou à duração do contato. Uma vez ultrapassados estes limites, ficam visíveis alterações nos processos bioquímicos bem como nas membranas celulares, com efeitos deletérios de curto e longo prazo nos órgãos dos organismos afetados e, consequentemente, no aparecimento de doenças em animais e seres humanos. Esta absorção e consequente retenção de metais pode derivar do contato direto do organismo com o poluente no ambiente em que habita – bioconcentração e de modo indireto através da ingestão de alimentos – biomagnificação. É de se esperar, portanto, que animais de hábito predador apresentem concentrações de metais mais elevadas do que suas presas (Andrade et al., 2010).

A EPA (Environmental Protection Agency) relata que "a toxicidade de certos compostos pode ser diminuída em algumas águas devido a diferenças na acidez, temperatura, dureza, dentre outros fatores. Em contrapartida, algumas características de águas naturais podem aumentar o impacto de certos poluentes." Desse modo, entende-se que o grau tóxico de um poluente varia de acordo com as variáveis físicas e químicas do ambiente contaminado, o qual pode ter seu efeito atenuado ou amplificado por um ou pela soma de fatores abióticos ambientais. Ambientes com potencial para amplificar os efeitos nocivos de tóxicos representam sistemas com alto risco para a biota, a qual utiliza de maneira direta e indireta dos recursos contaminados (Babich and Stotzky, 1982). Para exemplificar, os mesmos autores relatam que a toxicidade de metais como o Cádmio (Cd), Cromo (Cr), Cobre (Cu), Berílio (Be), Chumbo (Pb), Níquel (Ni), e Zinco (Zn) está diretamente ligada ao nível de dureza em águas doces: quanto mais alto o grau de dureza, menor a dose desse metal que é necessária para que os efeitos deletérios comecem a se manifestar.

Estes contaminantes geram, em última instância, intoxicações severas pelo consumo gradual da água e dos alimentos contaminados por estes subprodutos agrícolas, que bioacumulam em diferentes níveis tróficos, tais como na gordura de peixes e crustáceos, bem como no leite de vacas, com consequências ainda maiores para animais que ocupam o topo de cadeias alimentares, como o ser humano (Corbi et al., 2006).

1.2.1 Cobre, Cádmio, Manganês e Mercúrio

Entre os elementos metálicos, mercúrio, manganês, cobre e cádmio são alguns dos principais contaminantes devido ao seu uso recorrente em processos industriais. A alta nocividade destes pode causar efeitos adversos para a saúde humana e animal quando presentes em níveis elevados (Pierre-Marie et al., 2011).

O cobre é um metal particularmente perigoso devido à sua ocorrência recorrente em despejos industriais e agrícolas, assim como pela sua eficiência na intoxicação de organismos dos mais variados grupos, através de danos severos aos processos fisiológicos (Joachim et al., 2017). Águas com teores de cobre acima de 2,5 mg/L apresentam sabor amargo, sendo que acima de 1 mg L^{-1} são observadas alterações na cor de louças e sanitários (CETESB, 2009).

Em baixas doses, o cobre é essencial às plantas e animais, todavia, concentrações na água de 20 mg/L ou um teor total de 100 mg L^{-1} por dia podem levar a graves intoxicações no ser humano, afetando, principalmente, o fígado. Para peixes, os efeitos nocivos deste metal são potencializados pelo contato direto do organismo com o meio que o circunda, sendo que as concentrações de 0,5 mg L^{-1} já são letais para trutas, carpas, bagres, dentre outras espécies de peixes; para microrganismos, a concentração de 1mg L^{-1} é letal. A portaria 518/04 estabelece que o padrão de potabilidade para o cobre é de 2 mg L^{-1} (CETESB, 2009).

Concentrações altas de cobre foram registradas em corpos d´água próximos a regiões com cultivo de cana-de-açúcar; um resultado possivelmente relacionado ao descarte inadequado de esgotos sanitários e industriais, assim como à lixiviação do solo em áreas agrícolas com o consequente carreamento de subprodutos agrotóxicos e compostos a base de metais. Estas atividades e processos de carreamento explicam o porquê de altas concentrações de cobre mesmo em córregos sem atividades agrícolas próximas, reiterando a importância da manutenção de matas ciliares, as quais atenuam a entrada de metais nos corpos d´água (Corbi et al., 2006)

Embora resultados laboratoriais indiquem que o manganês tem um impacto ambiental reduzido quando comparado com outros metais (Harford et al., 2015), sua toxicidade pode ser aumentada por fatores físicos e químicos (Sanders et al., 1998) e pela ação conjunta com outros metais uma vez que este metal é um co-fator enzimático antioxidante essencial de várias reações bioquímicas (Martin et al., 2008, Michalke e Fernsebner, 2014). O manganês e seus derivados são utilizados tanto em indústrias do aço, ligas metálicas, baterias, vidros, oxidantes para limpeza, fertilizantes, dentre tantos outros usos, o que faz do manganês um metal com potencial poluidor muito grande devido às suas diversas fontes de contaminação. Mesmo sendo um metal com ocorrência natural em corpos d´água superficiais e subterrâneos (com concentrações de até 1,0 mg L^{-1}, embora geralmente seja encontrado em quantidades inferiores a 0,2 mg/L) observa-se um aumento nos teores deste metal devido às atividades antrópicas, o que confere à agua uma coloração negra. O manganês é um elemento essencial para muitos organismos, inclusive para o ser humano, os quais assimilam este metal por meio da alimentação. A portaria 518/04 estabelece em 0,1 mg L^{-1} o limite padrão de consumo do manganês pelos seres humanos (CETESB, 2009).

A biodisponibilidade deste metal depende fortemente de sua especiação, sendo que a maior parte do manganês encontrado em águas naturais está presente em suspensão, o que acarreta a eliminação deste metal da coluna d´água através da sedimentação e previne seus efeitos em níveis tóxicos. Há pouca informação acerca da toxicidade deste metal na literatura ecotoxicológica, majoritariamente devido à sua baixa toxicidade, embora níveis excessivos deste metal possam levar a doenças como o manganismo, semelhante à doença de Parkinson, tais como a CL50 (96h) de 1,723g L^{-1} para juvenis de *Oreochromis mossambicus* e o desencadeamento de anemia para altas concentrações de manganês em peixes (4,43mg L^{-1}) (Sanders et al., 1998).

Quanto ao cádmio, comumente associado aos fertilizantes fosfatados (Pelli L. Howe et al., 2014[a]), foram observados efeitos tóxicos no fígado, rins, pulmões e pâncreas, em animais. Este metal se acumula em organismos aquáticos através da cadeia alimentar, afetando ou mesmo inibindo os processos reprodutivos (Pelli L Howe et al., 2014[b]). O cádmio substitui o magnésio, zinco e cálcio em alguns processos metabólicos e pode causar câncer, se inalado (Sánchez-Bayo, 2011). A Portaria

518/04 considera a concentração de 0,005 mg L^{-1} em água de abastecimento como limite máximo permissível (CETESB, 2009).

O cádmio apresenta alta persistência ambiental e é amplamente distribuído em ambientes aquáticos. Ele entra nos ecossistemas por meios antropogênicos e naturais, pois é usado como componente da fundição, fabricação de baterias, tintas e fertilizantes (Burger, 2008; Dolagaratz Carricavur et al., 2018) De modo geral, águas não contaminadas apresentam concentração de cádmio inferior a 1 µg L^{-1}, enquanto a água considerada potável para o consumo humano possui valores entre 0,01 e 1 µg L^{-1}, valores estes sujeitos a aumentos devido a impurezas em tubulações galvanizadas, soldas e acessórios metálicos (CETESB, 2009).

O mercúrio, um metal persistente, tóxico e altamente bioacumulável, com grande mobilidade através da sua forma gasosa (Buch et al., 2017), pode induzir doença de Parkinson e danos irreparáveis ao fígado e aos rins (Andrade et al., 2010), apresentando graves efeitos cumulativos e danos cerebrais em seres humanos e peixes, bem como efeitos graves no crescimento, reprodução e sobrevivência de invertebrados, sendo que os Oligochaeta apresentam alto fator de bioacumulação (BAF) (Buch et al., 2017). Este metal se liga a grupos sulfeto em proteínas e suas formas organomercuriais são muito mais tóxicas devido à sua penetração nos tecidos e à contaminação do sistema nervoso (Sánchez-bayo, 2011). Nos corpos d'água brasileiros, as concentrações de mercúrio são geralmente inferiores a 0,5 µg L^{-1}, e o padrão aceitável para consumo estabelecido pela Portaria 518/04 é de 0,001 mg L^{-1}. (CETESB, 2009)

O efeito tóxico de metais em geral em animais aquáticos depende de uma séries de atributos físicos e químicos da água, como a dureza, alcalinidade, pH, matéria orgânica dissolvida, sólidos suspensos, dentre outras variáveis que podem ocasionar diferentes especificações químicas nos metais (Erickson et al., 1996).

1.3 O impacto ambiental dos agrotóxicos

Os pesticidas constituem um grupo de substâncias na maior parte sintetizadas artificialmente e utilizadas na agricultura para controlar pragas e aumentar a produção. Embora protejam as culturas agrícolas, o uso excessivo e incorreto dos agrotóxicos pode representar riscos para a saúde dos organismos direta ou indiretamente relacionados a estas culturas (Caldas et al., 2011; Rohr et al., 2012). Estes compostos foram identificados como um dos principais fatores para o declínio e extinção de inúmeras espécies, uma vez que podem causar mortalidade, deficiências no crescimento e desenvolvimento além de anormalidades nos mais distintos grupos de organismos (Jayatillake et al., 2011). Devido aos seus efeitos tóxicos sobre os organismos não-alvo, a maioria dos pesticidas produz efeitos deletérios na biota e no funcionamento dos ecossistemas. Embora a pulverização acidental não represente grande risco aos ecossistemas aquáticos, a lixiviação e o escoamento (Figura 3) representam um grave problema aos corpos d´água com prejuízos sistêmicos de longo prazo (Dobsikova, 2003). Quando a exposição a um inseticida ocorre em níveis abaixo daqueles que causam mortalidade, os indivíduos afetados podem sofrer os chamados efeitos subletais, que não estão relacionados ao modo específico de ação desse inseticida, e que podem levar a danos como a diminuição na produção de gametas, deformidades fisiológicas, retardo da maturidade sexual, dentre outros parâmetros do ciclo de vida (Sánchez-Bayo, 2012).

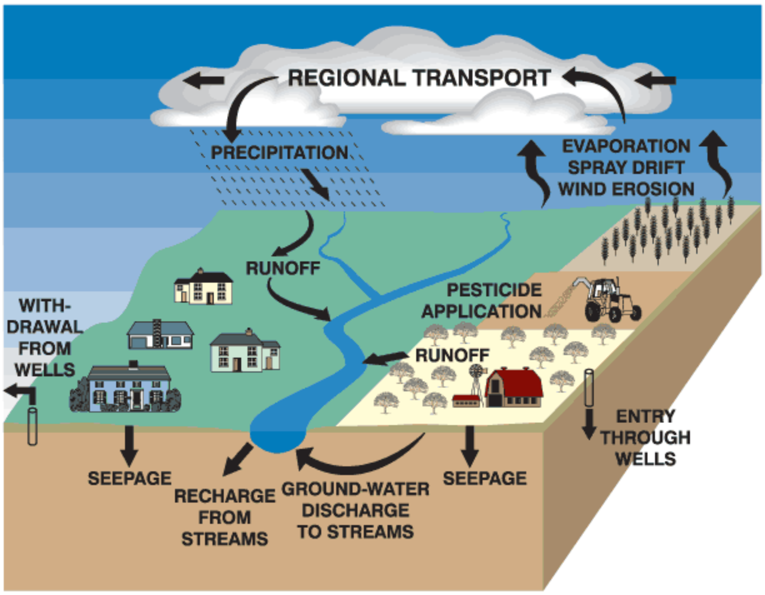

Figura 3. Trajeto dos pesticidas durante o ciclo hidrológico Fonte: Eddy-Miller 2004.

O carbofurano (Figura 4A) (2,3-dihydro-2,2-dimethylbenzofuran-7-ylmethylcarbamate) é um inseticida, nematicida e acaricida sistêmico de amplo espectro utilizado em todo o mundo. Como resultado de seu uso generalizado, o carbofuran foi detectado em águas continentais, de superfície e de chuva, em solos, ar, alimentos e associado à própria biota (Dobsikova, 2003). Este pesticida do grupo dos carbamatos é amplamente utilizado em campos de arroz, algodão, café, cana-de-açúcar, feijão e milho (Mansano et al., 2016). Um grave problema deste pesticida é o fato de não ser ecologicamente seletivo, o que pode representar uma séria ameaça para a sobrevivência de espécies não-alvo, dentro ou fora da área agrícola. O carbofurano se liga irreversivelmente à enzima acetil-colinesterase, inibindo a sua ação sobre um metabólito importante da acetilcolina, um dos motivos pelos quais o carbofurano é considerado altamente tóxico para a biota terrestre e, principalmente, para a aquática (Moreira et al., 2015)

O diuron (Figura 4B) [3-(3,4-diclorofenil)-1,1-dimetiluréia] é um herbicida de amplo espectro, pré e pós-emergente para uso contra ervas daninhas de folhas largas e gramíneas anuais(Giacomazzi and Cochet, 2004). Este produto químico apresenta moderada solubilidade em água e interrompe a fotossíntese inibindo a produção de oxigênio e bloqueia o transporte de elétrons em nível do fotosistema II e a transferência de energia luminosa (ENSR, 2005). O diuron é um dos pesticidas mais frequentemente encontrados em ecossistemas de água doce. É amplamente utilizado para o cultivo de cana-de-açúcar no Brasil e entra no solo também via fertilização (Freitas et al., 2016; Rodrigues et al., 2015). Embora as concentrações de diuron no ambiente dificilmente sejam elevadas a ponto de causar a mortalidade de organismos heterotróficos, concentrações subletais deste pesticida podem alterar vários parâmetros individuais através de efeitos sobre neurotransmissores, hormônios, resposta imunológica, reprodução, fisiologia, morfologia ou comportamento dos indivíduos (López-Doval et al., 2014).

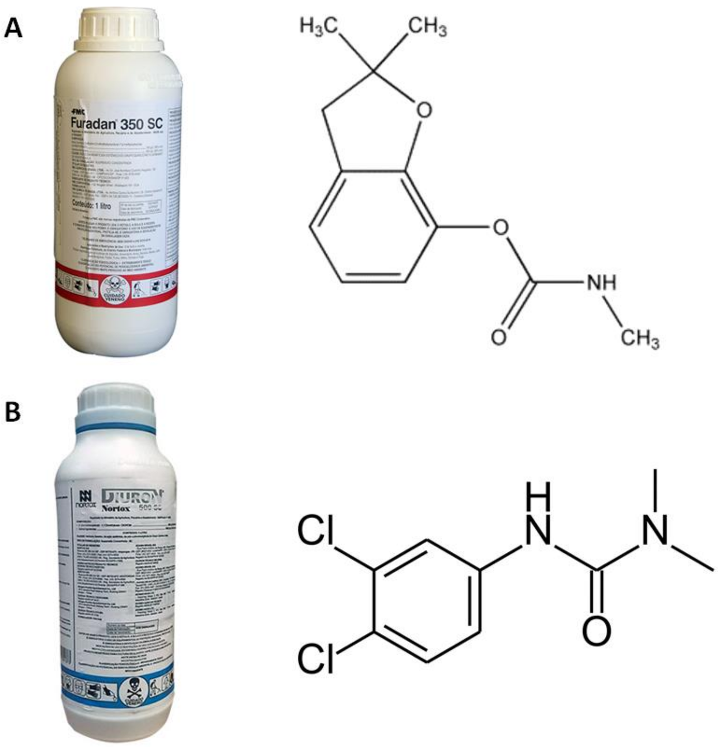

Figura 4. Fórmula estrutrural do carbofurano (A) e do diuron (B) e a imagem de seus respectivos produtos comerciais utilizados nos testes de toxicidade. Fonte: ChemSpider, 2018.

1.4 A toxicidade das misturas

A predição dos efeitos ecossistêmicos dos poluentes vê-se desprovida de sentido prático quando não considera a ação combinada de tais substâncias. Os poluentes presentes em um corpo hídrico não agem de maneira isolada e, portanto, a análise integrada dos componentes químicos do sistema analisado se faz necessária quando queremos embasar os estudos ecotoxicológicos na realidade local (Meyer et al., 2015). Atualmente, os parâmetros utilizados quando da análise de poluentes considera a atuação isolada dos compostos tóxicos, uma abordagem um tanto ultrapassada e por diversas vezes criticada por distintos autores, que ressaltam a necessidade de estudos das misturas tóxicas como pedra angular destas análises (Enserink et al., 1991).

Quando se utilizam substâncias tóxicas isoladas como referência para os efeitos adversos em uma população ou comunidade natural, depara-se com alguns problemas básicos: substâncias químicas, sejam elas metais ou compostos mais complexos, tais como agrotóxicos, não agem de maneira isolada no ambiente (Wu et al., 2016). Desse modo, como avaliar o mecanismo deletério de um corpo hídrico sobre as populações naturais se estamos levando em conta apenas uma de suas variáveis? Como desprezar os efeitos conjuntos, sejam eles antagônicos, aditivos ou sinérgicos e extrapolar os dados destas pesquisas para comunidades naturais (Jak et al., 1996)?

Embora os elementos metálicos isolados nem sempre tenham efeitos deletérios sobre os organismos, a contribuição tóxica destas espécies químicas para as misturas pode ser significativa para os organismos expostos, devido ao potencial de interações físicas e químicas e/ou biológicas (PORTER et al., 1993). Os compostos químicos com modos de ação semelhantes tendem a ter um efeito aditivo, como se observa para algumas misturas químicas orgânicas e inorgânicas, embora, para os metais, tais interações possam ser sinérgicas quando a soma das partes excede o resultado tóxico das espécies analisadas isoladamente, ou mesmo antagônico, quando a toxicidade da mistura diminui; outros tipos de interações podem ocorrer com a mesma combinação de substâncias tóxicas de acordo com os níveis ou proporção dos componentes da mistura (CETESB, 2015; FREITAS et al., 2014; JAK et al., 1996).

Estudos realizados por ENSERINK et al. (1991) demonstraram que mesmo quando concentrações isoladas de metais não desencadearam efeitos sobre o processo reprodutivo de *Daphnia magna*, a ação conjunta destes metais apresentou efeitos tóxicos para o organismo, sendo que a explicação teórica dada pelos autores é de que a ação conjunta e aditiva de agentes tóxicos, e de modo particular dos metais, têm origem no fato destes agirem de maneira qualitativamente semelhante, ou seja, com um modo de ação similar, que se potencializa com o aumento da concentração.

1.5 Testes Ecotoxicológicos com invertebrados bentônicos

A bioacumulação de metais em invertebrados aquáticos tem efeitos ainda maiores quando comparados aos efeitos causados em vertebrados (Costa et al., 2008), devido ao seu tamanho, contato direto e constante com o poluente e menor complexidade desses organismos, levando a deformações individuais, taxas de fertilização e/ou crescimento reduzidas ou ausentes e até mesmo a morte de populações inteiras (Depledge and Rainbow, 1990; Goodyear and McNeill, 1999; Hudspith et al., 2017).

Os organismos bentônicos apresentam importância singular em meio às investigações ecotoxicológicas, visto que funcionam como indicadores de qualidade do sedimento presente nos sistemas aquáticos, componente de integração dos processos de um sistema aquático, tais como a deposição de metais e organoclorados e cuja análise pode indicar contaminantes que não permanecem solúveis em águas superficiais (Corbi et al., 2006). Todavia, o uso de espécies bentônicas como organismos-teste em Ecotoxicologia não implica necessariamente no uso de sedimento durante a realização dos testes, uma vez que algumas análises primam pelo entendimento dos efeitos diretos da água contaminada sobre os organismos, os quais não passam todo ciclo de vida dentro do sedimento. Desse modo, em alguns estudos são utilizados ínstares livre-natantes que não são estressados pela ausência de sedimento e assim possibilitam uma análise da referida substância sem que o sedimento atenue sua toxicidade (OECD, 2011).

Os oligoquetos foram amplamente utilizados como organismos teste nos últimos anos para estudos ecotoxicológicos devido à sua ocorrência cosmopolita, fácil cultivo, grande biomassa em comparação com outras espécies de invertebrados aquáticos e sua importância no ciclo da matéria orgânica. No campo, eles são recorrentemente expostos a substâncias tóxicas por alimentos ou por contato corporal com sedimentos ou com água (Corbi et al., 2015; Gomes et al., 2017; Smith et al., 1991). Eles foram amplamente utilizados em ensaios de toxicidade de metal por sua capacidade de bioacumular grandes quantidades desses compostos ou modificar formas metálicas químicas durante o consumo, metabolismo e excreção (Helling et al., 2000; Homa et al., 2003). Apesar da importância e da abundância atestadas, algumas poucas espécies de oligoquetas aquáticos são utilizadas em estudos toxicológicos, tais como: *Tubifex tubifex* (Müller) (Naididae), *Lumbriculus variegatus* (Müller) (Lumbriculidae), *Pristina leidyi* Smith (Naididae) e *Branchiura sowerbyi*. Entre os oligoquetos, as espécies de Naididae são consideradas espécies importantes na transferência de energia e toxinas de níveis tróficos baixos para altos, pois são alimentos para peixes e espécies imaturas de invertebrados aquáticos (Corbi et al., 2015).

1.6 Justificativa

Existe comparativamente uma falta de estudos sobre espécies nativas de oligoquetos e de muitos outros grupos de invertebrados da região Neotropical constituindo, por exemplo, uma grande lacuna de informações científicas sobre distribuição geográfica, aspectos biológicos do ciclo de vida e, mais especificamente, de ensaios ecotoxicológicos com invertebrados de água doce (Gomes et al., 2017). Como a maioria dos estudos ecotoxicológicos são realizados para regiões temperadas, espécies e parâmetros físicos e químicos são representativos daquela realidade. Muitos estudos realizados em regiões tropicais são orientados por esses dados para a construção e a condução dos experimentos, o que pode talvez resultar em incongruências nos resultados obtidos. A partir deste possível desajuste de informações e da falta de estudos com espécies e parâmetros tropicais, é necessário e desejável encontrar organismos-teste mais representativos dessas realidades regionais, alguns dos quais já foram demonstrados por vários estudos recentes como mais sensíveis do que espécies típicas de clima temperado (Buch et al., 2017; Daam and Rico, 2016; Ghose et al., 2014; Pelli L Howe et al., 2014; Pelli L. Howe et al., 2014; Mansano et al., 2016; Silvano and Begossi, 2016)

2. Objetivos

2.1 Objetivo Geral

O objetivo do presente estudo foi avaliar os efeitos dos metais cobre, manganês, cádmio e mercúrio, isolados e em misturas, para duas espécies nativas de Oligochaeta, *Dero furcatus* e *Allonais inaequalis*, ambas de ocorrência comum nas águas doces brasileiras. Além disso, objetivou-se comparar a sensibilidade das espécies selecionadas com à de outros organismos-teste de diferentes grupos taxonômicos pertencentes à biota de água doce, para encontrar organismos-teste adequados para estudos ecotoxicológicos que nos permitam prever os impactos de contaminantes em ecossistemas aquáticos, utilizando-os como indicadores da qualidade ambiental e da saúde dos mesmos em regiões tropicais. Além disso, objetivou-se avaliar os efeitos tóxicos crônicos dos referidos metais e das formulações comerciais do carbofurano e do diuron. Para tanto investigou-se os efeitos sub-letais destes compostos no ciclo de vida dos anelídeos testados.

2.2 Objetivos Específicos

- Avaliar a toxicidade dos metais cádmio, cobre, manganês e mercúrio e dos pesticidas carbofurano e diuron a duas espécies de invertebrados;

- Avaliar a toxicidade aguda isolada e de misturas dos metais aos organismos-teste selecionados;

- Avaliar a toxicidade crônica para *Allonais inaequalis* por meio de parâmetros do ciclo de vida, quando expostos aos metais e aos pesticidas;

- Avaliar a sensibilidade das espécies quando comparadas a outras espécies do mesmo e de outros grupos taxonômicos da biota aquática.

2.3 Hipóteses

- Espécies nativas de Oligochaeta tropicais selecionadas neste estudo podem ser utilizadas na avaliação da toxicidade de contaminantes em testes ecotoxicológicos;

- Os compostos metálicos sulfato de cobre e sulfato de manganês, assim como o cloreto de mercúrio e cloreto de cádmio, tanto isolados como em mistura, causarão efeitos deletérios aos oligoquetos nativos;

- As misturas testadas apresentarão efeitos sinérgicos sobre ambas espécies nativas de Oligochaeta;

- Os compostos metálicos, bem como os pesticidas carbofurano e diuron causarão alterações em parâmetros do ciclo de vida da espécie *Allonais inaequalis*, como o crescimento dos organismos e a taxa de sobrevivência;

- Ambas as espécies nativas de Oligochaeta selecionados serão mais sensíveis do que espécies de região temperada recomendadas em protocolos padronizados mundialmente para a avaliação de toxicidade dos metais e pesticidas avaliados.

3. Materiais e Métodos

3.1 Organismos e Condições de cultivo

Indivíduos de *Allonais inaequalis* Stephenson, 1911 (Oligochaeta: Naididae) (Figura 5) foram obtidos a partir de culturas-estoque mantidas por mais de 24 meses no Departamento de Hidráulica e Saneamento da Universidade de São Paulo (São Carlos, Brasil (Corbi et al., 2015) e cultivadas no Laboratório de Ecotoxicologia do Departamento de Ecologia e Biologia Evolutiva da Universidade Federal de São Carlos (UFSCar - São Carlos, Brasil). Os indivíduos do oligoqueto *Dero furcatus* (Oligochaeta: Naididae) (Figura 6) foram coletados em cultivos na Estação Experimental de Aquicultura do Departamento de Hidrobiologia da Universidade Federal de São Carlos, SP (Figura 7), realizados em tanques com água doce localizados no campus da UFSCar (21°58'54,7" S 47°52'39,3" W), sob elevadas temperaturas e abundante biomassa algal (a chlorophyta *Chlorella sorokiniana*). Trazidos ao laboratório, estes organismos foram mantidos para aclimatação (Figura 8) durante uma semana antes dos testes de toxicidade serem realizados, de acordo com os parâmetros estipulado pelo protocolo da OECD (2008) protocolo nº 315 para estudos de Bioacumulação de compostos tóxicos em oligoquetos bentônicos. As culturas foram mantidas sob fotoperíodo controlado (16 h Luz: 8h Escuro) e temperatura constante de 22 °C ± 2 °C. Os oligoquetos foram cultivados em água reconstituída com características conhecidas de oxigênio dissolvido, dureza (90,0-400,0 mg $CaCO_3$ L^{-1}), condutividade (150,0-350,0 μScm^{-1}), temperatura e pH (6,0-9,0), medidos semanalmente, conforme recomendado pelas Diretrizes da OECD para o Teste de Químicos (OECD, 2008). Os oligoquetas foram alimentados a cada sete dias com 20 mL de alimento de peixe Tetramim®, preparados com 5 g em dois litros de água destilada.

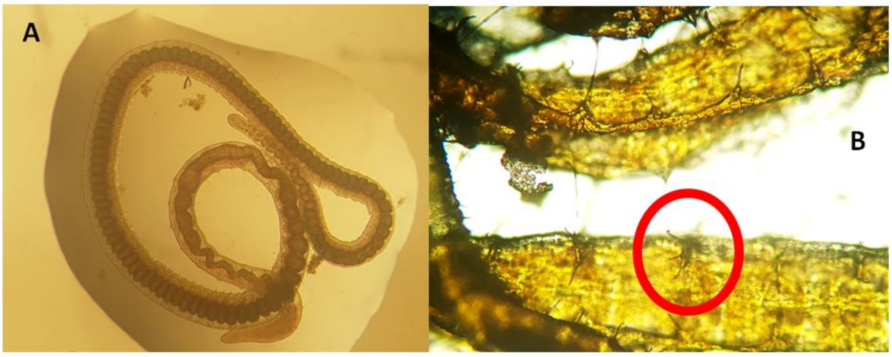

Figura 5. Imagens obtidas em microscopia óptica do oligoqueta *Allonais inaequalis.* A: Visão geral do organismo. B: Cerdas utilizadas para a identificação. Fonte: harpercollege.edu.

Figura 6. Imagens obtidas em microscopia óptica do oligoqueta *Dero furcatus.* Fonte: www.harpercollege.edu.

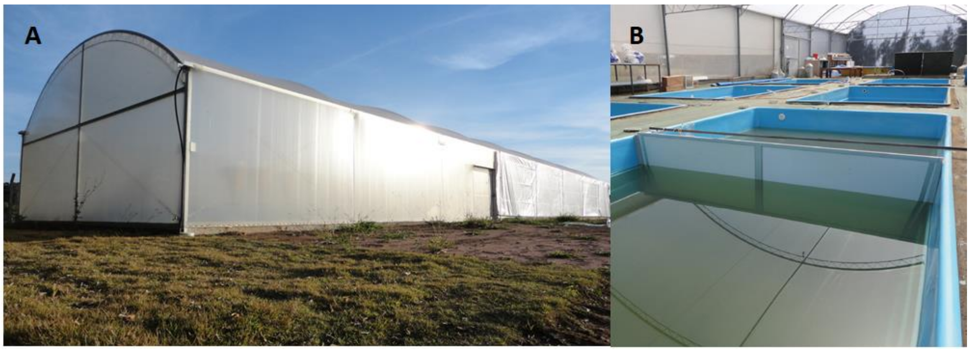

Figura 7 - Vistas externa (A) e interna (B) da Estação Experimental de Aquicultura do Departamento de Hidrobiologia da Universidade Federal de São Carlos – DHb/UFSCar Foto: Yeda C. Paccagnella, 2011.

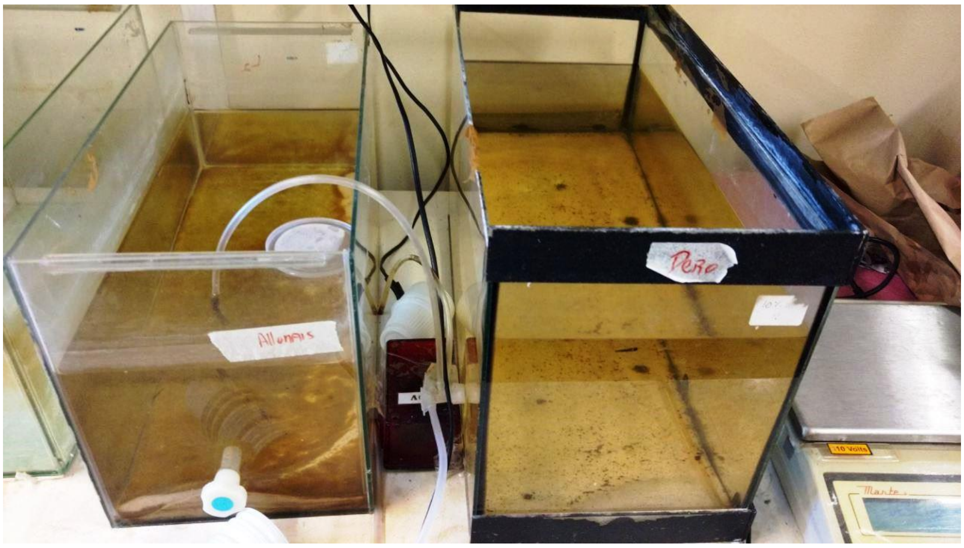

Figura 8. Aquários utilizados para o cultivo das espécies de Oligochaeta *Dero furcatus* e *Allonais inaequalis*. Foto: Daniele Cristina Schiavone, 2018.

3.2 Substâncias químicas e Soluções teste

Foram preparadas soluções-estoque (Figura 9) de cloreto de cádmio ($CdCl_2$ - CAS nº: 10108-64-2 - Carlo Erba), cloreto de mercúrio ($HgCl_2$ - CAS nº: 7487-94-7 - ACS Merck) e sulfato de cobre ($CuSO_4$ - CAS nº: 7758-98-7), 10 mg L^{-1} para os três compostos imediatamente antes dos testes de toxicidade, bem como uma solução-estoque de 100 mg L^{-1} de sulfato de manganês (MnSO4 – CAS nº: 7785-87-7). As concentrações nominais das substâncias testadas foram obtidas por diluição da solução-estoque em meio de cultura. Os testes preliminares realizados para determinar o intervalo de concentrações experimentais, bem como testes de toxicidade, seguiram o protocolo emitido pela OECD (2008).

Figura 9. Soluções-estoque preparadas com cloreto de cádmio, cloreto de mercúrio, sulfato de cobre e sulfato de manganês (frente) e os compostos utilizados no seu preparo (fundo). Foto: Daniele Cristina Schiavone, 2018.

A faixa de sensibilidade das espécies foi testada mensalmente (20 testes) utilizando sulfato de cobre como substância de referência, a fim de certificar as condições de saúde do Oligochaeta testado e, desse modo, a validade dos testes de toxicidade. Os parâmetros de qualidade da água - temperatura, dureza da água, condutividade e pH - também foram medidos no início e no final dos testes de toxicidade para cada ensaio realizado.

3.3 Testes de Toxicidade Aguda: compostos isolados e em mistura

Para os testes de toxicidade aguda (96h de exposição) (Figura 10) com cobre, manganês, cádmio e mercúrio, foram estabelecidas quatro repetições para cada tratamento de metal, com cinco diferentes concentrações de cada composto, além de um controle. Para certificar a reprodutibilidade dos valores de toxicidade e levar em consideração a variabilidade dos resultados, foram realizados cinco testes de toxicidade aguda definitiva para os quatro compostos. Os testes foram realizados em copos de plástico com seis adultos para testes isolados e cinco adultos para testes de mistura (tamanho médio: 7,8 mm - *A. inaequalis*, 7,0 mm - *D. furcatus*) em 10 mL da solução de teste ou 10 mL da água reconstituída, para o controle. Uma vez realizados os testes preliminares, foram estabelecidas as faixas de concentrações experimentais para cada composto e a partir disto foram realizados testes de toxicidade aguda para as seguintes concentrações nominais: 0,005; 0,01; 0,02; 0,04; 0,06 e 0,08 mgL^{-1} de sulfato de cobre, 0,2; 0,4; 0,8; 1,6 e 3,2 mgL^{-1} de cloreto de cádmio e 0,05; 0,1; 0,2; 0,3 e 0,4 mgL^{-1} de cloreto de mercúrio para as duas espécies de Oligochaeta. Para o sulfato de manganês, foram utilizadas as seguintes concentrações nominais: 27,5; 28,4; 29,4; 30,4; 31,4 e 32,4 mgL^{-1} para a espécie *Allonais inaequalis* e 17,5; 18,4; 19,4; 20,4; 21,4 e 22,4 mgL^{-1} para a espécie *Dero furcatus*. Os experimentos foram mantidos sob temperatura constante de 22 ± 2 °C, em incubadora, no escuro e sem alimentação. As leituras foram realizadas, após 48h e 96h de exposição. Para isso os indivíduos experimentais foram observados sob um estereomicroscópio da marca Zeiss, em aumento de até 50X, e os números de organismos mortos foram contados e utilizados para calcular a concentração letal média (96-h LC_{50}).

Figura 10. Teste de toxicidade aguda (96h) realizado com o organismo-teste *Dero furcatus.*
Foto: Mariana Miguel, 2016.

Para avaliação do efeito combinado de cloreto de cádmio e cloreto de mercúrio, os indivíduos foram expostos simultaneamente em testes individuais de cada metal e em testes com combinações desses dois compostos, através de um desenho fatorial para toxicidade binária (25 combinações). Os métodos de exposição aos compostos e os parâmetros analisados foram os mesmos já descritos anteriormente para os bioensaios de toxicidade aguda para os compostos de metais isolados.

3.4 Testes de Toxicidade Crônica

Os testes de toxicidade crônica foram realizados durante 12 dias com os metais sulfato de cobre, sulfato de manganês, cloreto de cádmio e cloreto de mercúrio e com as formulações comerciais dos pesticidas carbofurano e diuron. Dez réplicas foram estabelecidas para cada tratamento, com um indivíduo em cada repetição e seis concentrações para cada composto, além do controle. Os testes para metais e para pesticidas foram realizados em copos de plástico com indivíduos juvenis (tamanho médio: 3,75 mm), em 10 mL de solução teste ou 10 mL de água reconstituída no caso do controle.

Para a determinação do gradiente de concentração, foi utilizado o LC_{20} obtido nos testes agudos para cada uma das referidas substâncias, com um fator de divisão 2 para o cálculo das concentrações inferiores. Foram utilizadas as seguintes concentrações nominais: 0,000625; 0,00125; 0,0025; 0,005; 0,01 e 0,02 mgL^{-1} de sulfato de cobre; 0,8625; 1,725; 3,45; 6,9; 13,8 e 27,6 mgL^{-1} de sulfato de manganês, 0,0141; 0,0281; 0,05625; 0,1125; 0,225 e 0,45 mgL^{-1} de cloreto de cádmio 0,003125; 0,00625; 0,0125; 0,025; 0,05 e 0,1 mgL^{-1} de cloreto de mercúrio, 0,02346; 0,046875; 0,09375; 0,1875; 0,375 e 0,75 mgL^{-1} de formulação comercial de carbofurano e 0,375; 0,75; 1,5; 3; 6 e 12 mgL^{-1} de formulação comercial do diuron.

As experiências com ambos, metais e pesticidas, foram mantidas no escuro a temperatura de 22 ± 2,5 °C. As leituras foram realizadas no dia inicial, no 3º, 5º, 8º, 10º e 12º dia (último dia). Os indivíduos testados foram observados sob um estereomicroscópio e o número de organismos mortos foi contado e o comprimento de cada organismo (em mm) foi registrado.

3.5 Curva de Distribuição da Sensibilidade das Espécies

As distribuições de sensibilidade de espécies (SSDs) foram construídas para comparação dos valores de toxicidade aguda das Concentrações Efetivas (96h CE_{50})

dos metais testados obtidas nos diversos testes realizados com os organismos-teste *Dero furcatus* e *Allonais inaequalis*. Assim, os resultados obtidos para a toxicidade dos compostos cloreto de cadmio, cloreto de mercúrio, sulfato de cobre e sulfato de manganês foram cada um comparados aos valores obtidos para cada um destes metais para outras espécies de invertebrados. Os dados de toxicidade para outros invertebrados foram compilados a partir do banco de dados ECOTOX da USEPA (http://cfpub.epa.gov/ecotox/, 2017), e complementado com dados da literatura aberta. As médias geométricas foram calculadas quando mais de um valor de toxicidade foi relatado para uma determinada espécie.

As distribuições log-normal de valores-limiar foram construídas utilizando-se o programa de computador ETX, versão 2.0 (Van Vlaardingen et al., 2004). O 5º percentual (concentração perigosa para 5% das espécies-HC5) e 50º percentual (concentração perigosa para 50% das espécies-HC50) com seus limites de confiança foram calculados com o referido software com base na metodologia descrita por Aldenberg e Jaworska (2000). Uma vez que o modelo assume uma distribuição log-normal dos dados, a log-normalidade foi testada com o teste Anderson-Darling incluído no pacote de software ETX, que foi avaliado no nível de significância de 5%.

3.6 Análise dos Dados

Para os testes de toxicidade aguda, os valores de $96hCE_{50}$ (intervalos de confiança de 95%) foram calculados pelo software gratuito PriProbit (versão 1.63). Em todos os testes estatísticos aplicados, as diferenças obtidas foram consideradas significativas quando $p \leq 0,05$.

Os dados obtidos para os testes de toxicidade com as misturas dos metais foram analisados comparando-se os dados observados com os efeitos combinados esperados pelos modelos de referência de adição de concentração (CA) e de ação independente (IA) usando a ferramenta MIXTOX (Jonker et al., 2005). Numa segunda etapa da análise dos dados, os modelos CA e IA foram estendidos conforme descrito por Jonker et al. (2005) e as funções de desvio, tais como interações sinérgicas / antagônicas, desvios dependentes da proporção de dose e do nível de dose foram modeladas pela adição de dois parâmetros ("a" e "b"), formando um estrutura aninhada. No desvio de sinergismo/antagonismo, o parâmetro "a" torna-se, respectivamente, negativo ou positivo. Para o desvio dependente da proporção da dose (DR), o valor do parâmetro " bDR ", além do parâmetro "a", indica que o desvio do modelo de referência é controlado pela composição da mistura. Para o desvio dependente do nível de dose (DL), o parâmetro " bDL " está incluído além de "a". Nesta função de desvio, o valor de "a" indica o desvio em altas e baixas doses e o valor de "bDL" indica em que nível da dose o desvio muda . Mais detalhes sobre essas funções de desvio podem ser encontrados em Jonker et al. (2005). Os dados foram ajustados a modelos conceituais e desvios, e o melhor ajuste foi escolhido pelo método de máxima verossimilhança. Quando um modelo de desvio estatisticamente mais descritivo foi identificado, o padrão de efeito foi deduzido diretamente dos valores dos parâmetros e o desvio máximo foi calculado em termos de nível de efeito (Freitas et al., 2014; Jonker et al., 2005).

Para os testes de toxicidade crônica realizados com os metais cobre, manganês, cádmio, mercúrio e os pesticidas carbofuran e diuron, tanto para o parâmetro de mortalidade quanto de tamanho, o teste de normalidade (Shapiro-Wilk) e a homogeneidade dos dados (Levene) foram verificados e as diferenças entre os tratamentos foram avaliados por análise de variância (ANOVA). Seguiu-se o teste pós-hoc Fisher LSD em caso de dados que atendessem aos critérios de normalidade e homoscedasticidade. Para os dados que não atendiam a esses requisitos, utilizaram-se o teste não paramétrico Kruskall-Wallis, seguido do teste pós-hoc Student-Newman-Keuls. Diferenças significativas entre os grupos foram aceitas em $p < 0{,}05$. As análises foram realizadas usando o software gratuito Statistica versão 7 (Statsoft, 2004).

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5. Resultados e Discussão

5.1. Freshwater Neotropical oligochaetes as native test species for the toxicity evaluation of cadmium, mercury and their mixtures

Highlights

- Mixture toxicity of two metals was evaluated to two Neotropical Oligochaeta.
- Mercury chloride toxicity to both oligochaetes was higher than cadmium chloride.
- *Dero furcatus* was twice more sensitive than *Allonais inaequalis* regarding cadmium chloride.
- *A. inaequalis* and *D. furcatus* were more sensitive than *Tubifex tubifex* for $CdCl_2$.
- Synergism occurred at high concentrations of these metals mixtures.

Abstract

The toxicity of metals, whether isolated or in mixtures, involves changes in biochemical processes as well as in cell membranes, which may lead to deleterious short- and long-term effects on the affected organisms. Among the metals, cadmium and mercury stand out due to their frequent use in industrial activities and bioaccumulation capacity, with high levels of residence in trophic chains. Benthic communities are particularly prone to metal pollution since metals may accumulate in sediments. The aim of this study was to evaluate the acute single and mixture toxicity of mercury and cadmium to two native species of epibenthic oligochaetes: *Allonais inaequalis* and *Dero furcatus*. In order to assess the potential of these species as bioindicators, we compared their sensitivity with that of other internationally used species by applying the species sensitivity distribution approach. The 96h LC_{50} for cadmium chloride was 627 µg L^{-1} and 364 µg L^{-1} for *A. inaequalis* and *D. furcatus*, respectively, evidencing that the latter species is almost twice as sensitive to this metal than *A. inaequalis*. For mercury chloride, the LC_{50} was 129 µg L^{-1} for *A. inaequalis* and 92 µg L^{-1} for *D. furcatus*. The sensitivities of the oligochaetes tested were superior or similar to other frequently used oligochaete test species such as *Tubifex tubifex* and *Lumbriculus variegatus*. The metal mixtures revealed synergism in general (*D. furcatus*) or at high doses only (*A. inaequalis*), implying a potentiation of their toxic effects when both metals co-occur in the environment. By comparing the derived toxicity values in this study with concentrations of cadmium and mercury measured in the field, it can be concluded that oligochaetes and other invertebrates are likely to be at risk when exposed to environmental relevant concentrations of cadmium and mercury, especially when they are both present.

Keywords: Ecotoxicology; Metals; Annelida; Tropical species; Species Sensitivity Distribution.

1. Introduction

The importance of several essential metals for some basic functions of life is evident, since they are basic constituents of enzymes, hormones, and thus responsible for metabolic processes maintenance, besides prevention of diseases (Andrade et al., 2010; CETESB, 2009; Sanders et al., 1998). Higher levels of metal compounds, however, may lead to undesirable side- effects to human health and the environment. The toxicity of metals is usually linked to its high nephrotoxic potential (Hodgson, 2004), mainly due to the bonding that these inorganic toxins tend to form with organic molecules, which can deactivate their functionalities (Sánchez-Bayo, 2012). Bioaccumulation of metals in aquatic invertebrates has been reported to have greater effects when compared to vertebrates (Costa et al., 2008) due to their smaller size, direct and constant contact with the pollutant, and lower complexity of these organisms, leading to individual deformation, reduced fertilization and/or growth rates and even death of an entire population (Depledge and Rainbow, 1990; Goodyear and McNeill, 1999; Hudspith et al., 2017).

The presence of metals in aquatic systems can, at least partially, be explained by typical natural processes, such as volcanic activities or soil erosion (Parelho et al., 2014). However, discrepant levels of aquatic metal concentrations are most probably a direct result of anthropic action, mainly by industrial waste disposal or contaminated soil from agricultural areas (Kelepertzis, 2014; Mansour and Sidky, 2002). The increasing use of metal-based fertilizers in sugarcane crops, one of the main monocultures in Brazil (Azania et al., 2009), culminated with significant contamination of surrounding aquatic ecosystems. Since metals can be absorbed by plant and animal tissues, they may affect non-target organisms throughout terrestrial and aquatic ecosystems and their interfaces (Corbi et al., 2006; Ming-Ho Yu, 2004).

Among metals, mercury and cadmium are some of the main contaminants due to their recurrent use in industrial processes. Their high toxicity may cause severe adverse effects to human and animal health when present at high levels (Pierre-Marie et al., 2011). Mercury has the potential to ensue severe effects on growth, reproduction and survival of invertebrates, as shown by its high bioaccumulation factor (BAF) in Oligochaeta (Buch et al., 2017). This metal binds to sulfide groups in proteins and its organic mercurial forms possess greater toxicity due to their penetration in tissues and the nervous system (Sánchez-bayo, 2011). Cadmium is commonly associated with phosphate fertilizers (Howe et al., 2014). Cadmium replaces magnesium, zinc and calcium in some metabolic processes and may as such exert toxic effects in liver, kidneys, lungs and pancreas (Sánchez-bayo, 2011). This metal may also accumulate in aquatic organisms and affect their reproductive processes (Howe et al., 2014). In general, uncontaminated waters have cadmium concentrations lower than 1 µg L^{-1} and the maximum cadmium level allowed for drinking water by the Brazilian government is 0.005 mg L^{-1} (CETESB, 2009).

Although exposure to single metals may not lead to side-effects on organisms, their mixtures may have significant toxic effects on organisms through potential physicochemical and/or biological interactions (Porter et al., 1993).

Oligochaetes have been widely used as test-organisms in recent years for ecotoxicological studies due to their cosmopolitan occurrence, easy cultivation, large biomass when compared to other species of aquatic invertebrates and its importance in organic matter cycling (OECD, 2008; EFSA, 2015). In the field, they are recurrently exposed to toxic substances either by food or by body contact with sediment or with water (Corbi et al., 2015; Gomes et al., 2017; Smith et al., 1991). They have been extensively used in metal toxicity assays for their ability to bioaccumulate large amounts of these compounds and to modify metal chemical forms during consumption, metabolism, and excretion (Helling et al., 2000; Homa et al., 2003). Despite their importance and abundance few aquatic oligochaete species have been used so far in toxicological studies, namely: *Tubifex tubifex* (Müller) (Naididae), *Lumbriculus variegatus* (Müller) (Lumbriculidae), *Pristina leidyi* Smith (Naididae) and *Branchiura sowerbyi* (e.g. EFSA, 2015; Lobo et al., 2016; OECD, 2008). Among oligochaetes, Naididae species are considered important links for transfer energy and toxins from low to high trophic levels, as they are food for fishes and immature species of aquatic invertebrates (Corbi et al., 2015).

In most tropical regions there is a lack of studies using native species, constituting a gap of scientific information regarding distribution mapping, biology, and, specifically, ecotoxicological assays (Gomes et al., 2017). Since most of the ecotoxicological studies have been carried out in temperate regions, physical and chemical parameters of the sediments, species and experimental conditions are representative of those regions, what may generate incongruences in the obtained results. Subsequently, it has often been pointed out that it is necessary to test indigenous organisms under conditions that are representative for tropical regions (Buch et al., 2017; Daam and Rico, 2016; Ghose et al., 2014; Pelli L Howe et al., 2014; Pelli L. Howe et al., 2014; Mansano et al., 2016; Silvano and Begossi, 2016).

The aim of the present study was to evaluate the toxicity of cadmium and mercury, isolated and in mixtures, to the two native oligochaetes *Dero furcatus* and *Allonais inaequalis*, which both commonly occur in Brazilian freshwaters (Cetesb, 2015, 2014). We also compared their sensitivity to the metals with that of other aquatic test organisms from different taxonomic groups and freshwater communities. Ultimately, this was done to evaluate the appropriateness of the two oligochaetes as sensitive test species for use in environmental risk assessments in Neotropical regions.

2. Materials and Methods

2.1. Test organism and culture conditions

Individuals of *Allonais inaequalis* Stephenson, 1911 (Oligochaeta: Naididae) were obtained from stock cultures maintained for more than 24 months at the Hydraulics and Sanitation Department of the University of São Paulo (São Carlos, Brazil) and cultivated in the Ecotoxicology Laboratory of the Department of Ecology and Evolutionary Biology, Federal University of São Carlos (UFSCar – São Carlos, Brazil). *Dero furcatus* individuals were isolated from freshwater concrete tanks at the UFSCar campus (21°58'54.7"S 47°52'39.3"W). They were acclimatized for a week before the start of the toxicity tests under the conditions outlined in OECD (2008): photoperiod: 16h light : 8h dark; temperature: 22 ± 2.5°C (Oximeter Hanna, USA, model HI9146). During this period, animals were kept in aquaria containing 10 L of reconstituted water with known conditions of hardness (90.0-400.0 mg $CaCO_3$ L^{-1} - as total calcium carbonate by titration method - Mackereth et al. 1978), conductivity (410 – 500 µScm^{-1} - Digimed Conductivity meter DO-48 model, Brazil), and pH (6.0-9.0 - Micronal pH meter model B371, Germany), which were measured weekly as recommended in OECD (2008). The oligochaetes were fed once a week with 20 mL

Tetramim® fish feed solution, which was prepared by adding 5 g Tetramim® to two liters of distilled water.

2.2. Chemicals and test solutions

Stock solutions of cadmium chloride ($CdCl_2$ – CAS no: 10108-64-2 – Carlo Erba) and mercury chloride ($HgCl_2$ – CAS no: 7487-94-7 – ACS Merck), 10 mg L^{-1} for both compounds, were prepared immediately prior to the toxicity tests. Nominal concentrations of the test substances were obtained by dilution of the stock solution with culture medium. Preliminary tests performed to determine the experimental concentration ranges as well as toxicity tests followed the protocol issued by OECD (2008).

Sensitivity of the organisms was tested monthly using copper sulfate as reference substance ($CuSO_4$ – CAS no: 7758-98-7), in order to certify the health conditions of the oligochaetes tested and hence the validity of the toxicity tests. Water quality parameters - temperature, water hardness, conductivity and pH - were measured at the start and at the end of each toxicity test.

2.3. Acute toxicity tests: isolated compounds and their mixtures

In the 96h acute toxicity tests with cadmium and mercury, four replicates for each metal treatment were established, six individuals in each replicate, and five concentrations of each compound, besides a control were tested. To certify the reproducibility of the toxicity values and take into account the variability of the results, five definitive acute toxicity tests were performed for both compounds. Tests were performed in plastic cups with six adults for isolated tests and five adults for mixtures tests (average size: 7.8 mm - *A. inaequalis*; 7.0 mm – *D. furcatus*) in 10mL of the test solution or 10mL of the reconstituted water, for control. Once the preliminary tests have been performed, the experimental concentrations range for each compound was established and acute toxicity tests were carried out according to the following nominal concentrations ranges: 0.2; 0.4; 0.8; 1.6 and 3.2 mgL^{-1} of cadmium chloride and 0.05; 0.1; 0.2; 0.3 and 0.4 mgL^{-1} of mercury chloride for both Oligochaeta.

Experiments were maintained at the temperature 22 ± 2 °C, in darkness and without food. Readings were performed, after 48h and 96h of exposure. Therefore, experimental individuals were observed under a stereomicroscope and the number of dead organisms was counted and used to calculate the mean lethal concentration (96-h LC_{50}).

In order to evaluate the combined effect of cadmium chloride and mercury chloride, individuals were exposed simultaneously in single tests of each metal and in tests with combinations of these two compounds, through a factorial design for binary toxicity (25 combinations). The compound exposure methods and analyzed parameters were the same as those previously described for isolated acute toxicity bioassays.

2.4. Data analysis

The acute toxicity tests 96-h LC_{50} values (95% confidence intervals) were calculated by PriProbit free software. In all statistical tests, difference was considered significant when $p \leq 0.05$.

Data from mixture toxicity tests were analyzed by comparing the observed data with the expected combined effects of concentration addition (CA) and independent action (IA) reference models using the MIXTOX tool (Jonker et al., 2005). In a second step of the data analysis, both CA and IA models were extended as described by Jonker et al. (2005) and deviation functions, such as synergistic/antagonistic interactions, dose-ratio and dose-level dependent deviations were modeled by the addition of two parameters (“a” and “b”, Table 1), forming a nested framework. In the synergism/antagonism deviation, the parameter “a” becomes, respectively, negative or positive. For dose-ratio dependent deviation (DR), the value of the parameter “bDR” in addition to the “a” parameter, indicates that the deviation from the reference model is controlled by the composition of the mixture. For dose-level dependent deviation (DL), the parameter “bDL” is included in addition to “a”. In this deviation function, the value of “a” indicates the deviation at low doses (i.e., a>0 = antagonism, and a<0 = synergism) and the value of “bDL” indicates at what dose level the deviation changes. More details on these deviation functions can be found at Jonker et al. (2005). Data were fitted to conceptual models and deviations, and the best fit was chosen by the maximum likelihood method. Where a statistically more descriptive deviation model was identified, the effect pattern was deduced directly from the parameter values (Table 1) and the maximum deviation was calculated in terms of effect level (Freitas et al., 2014; Jonker et al., 2005).

Table 1. Interpretation of the additional parameters ("a" and "b") that define the functional form of the standard deviations from the independent action model (IA); adapted from Jonker et al. (2005).

Deviation	Parameter "a"	Parameter "b"
Synergism/ Antagonism (S/A)	**a > 0** - antagonism **a < 0** - synergism	
Dose ratio-dependent (DR)	**a > 0** - antagonism, except for those proportions of mixtures where a value of significant negative b indicates synergism **a < 0** - synergism, except for those proportions of mixtures where a significant positive value of b indicates antagonism	**b_i > 0** - antagonism where the toxicity of the mixture is mainly caused by the toxic agent *i* **b_i < 0** - synergy where the toxicity of the mixture is mainly caused by toxic *i*
Dose level-dependent (DL)	**a > 0** - low dose level antagonism and synergy in high dose level **a < 0** - low dose level synergism and antagonism in high dose level	**b_{DL} > 2** - change in level of dose lower than EC_{50} b_{DL} = 2 - change in EC_{50} **1 < b_{DL} < 2** - change in level of dose greater than EC50 **b_{DL} < 1** - no change, but the magnitude of S/A is dependent on the level of effect

2.5. Species sensitivity distribution

Species sensitivity distributions (SSDs) were constructed to compare acute LC_{50} values obtained from the *Dero furcatus* and *Allonais inaequalis* tests evaluating cadmium chloride and mercury chloride with corresponding values for other invertebrate species. Toxicity data for invertebrates were compiled from the USEPA ECOTOX database (http://cfpub.epa.gov/ecotox/, 2017), supplemented with data from the open literature. Geometric means were calculated when more than one toxicity value was reported for a given species. Log-normal distributions of threshold values were constructed using the ETX computer program, version 2.0 (Van Vlaardingen et al., 2004). The 5th percentile (hazardous concentration for 5% of the species-HC_5) and 50th percentile (hazardous concentration for 50% of the species-HC50) with their confidence limits were calculated with the referred software based on the methodology described by Aldenberg and Jaworska (2000). Since the model assumes a log-normal distribution of the data, log-normality was tested with the Anderson-Darling test included in the ETX software package, which was evaluated at the 5% significance level.

3. Results and discussion

3.1. Test performance

Survival rates greater than 90% were observed in the controls at the end of all acute toxicity tests performed. The water chemistry parameters in all treatments during the course of all tests were as follows (min – max values): pH: 7.1 - 7.6, with variation throughout the test of less than 1.0 unit; water temperature: 22.6 - 24.5 ºC; conductivity: 416 - 457 µScm^{-1}; water hardness: 174 - 202 mg $CaCO_3$ L^{-1}. Therefore, all criteria for test validation established by OECD (2008) were attended. In total, twenty reference toxicity tests with copper sulfate were conducted with *A. inaequalis and D. furcatus* (Figure 1 and 2). These tests revealed 96h-LC_{50} values of 25.8 µg L^{-1} (95% CI: 23.4 - 28.1 µg L^{-1}) and 31.4 µg L^{-1} (25.0 - 37.8 µg L^{-1}), respectively. These toxicity values are at the lower than the of reported 96h-LC50 values for commonly tested oligochaetes such as *Branchiura sowerbyi* (80 µg L^{-1}; Das and Das, 2005), *Lumbriculus variegatus* (320 µg L^{-1}; Ewell et al., 1986) and *Tubifex tubifex* (30 – 80 µg L^{-1}; Maestre et al., 2009). To the best of our knowledge, no toxicity data are available for our test species evaluating copper sulfate. However, these reference values will enable comparing the sensitivity and hence health conditions of these organisms in future toxicity tests with these two taxa.

Table 2. Mean values of LC_{50} and standard deviation (SD) obtained in acute toxicity tests of two different metals to the oligochaete species *Dero furcatus* and *Allonais inaequalis*.

Compounds tested	***Dero furcatus*** **96h LC_{50} (µg L^{-1})**	***Allonais inaequalis*** **96h LC_{50} (µg L^{-1})**
Cadmium Chloride	364.158 ± 10.957	626.684 ± 18.081
Mercury Chloride	91.582 ± 2.982	129.189 ± 2.399

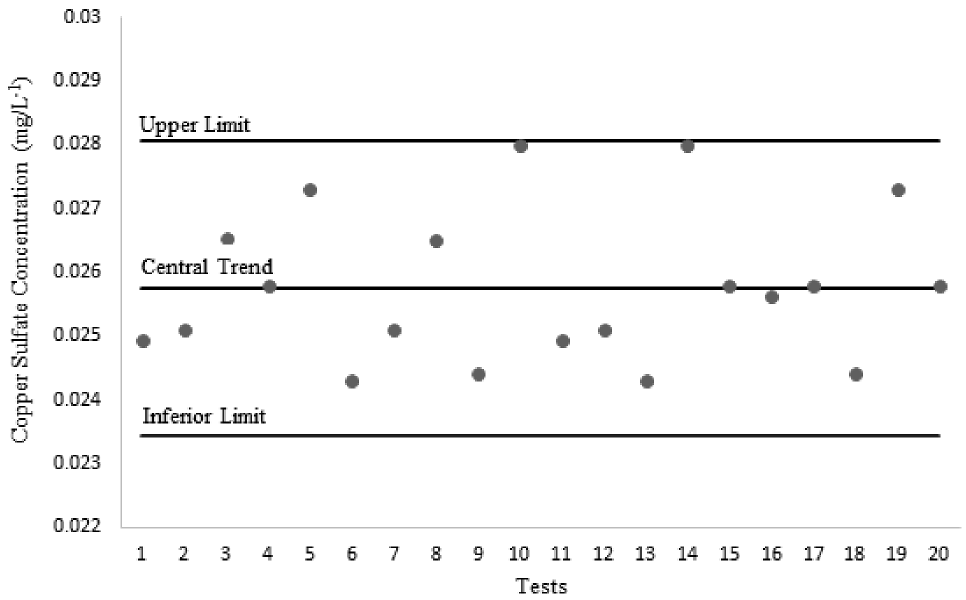


Figure 1. Range of sensitivity of *Allonais inaequalis* to copper sulfate based on the results of 20 acute toxicity tests. The upper and lower limits (95 % confidence intervals) were 0.0281 and 0.0234 mg L^{-1} of copper sulfate.

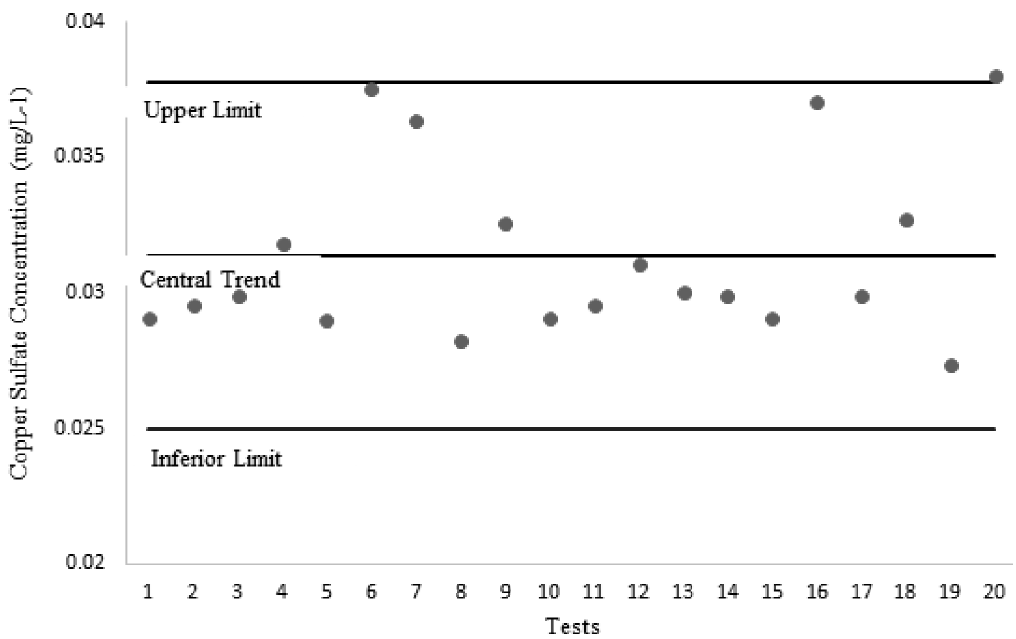
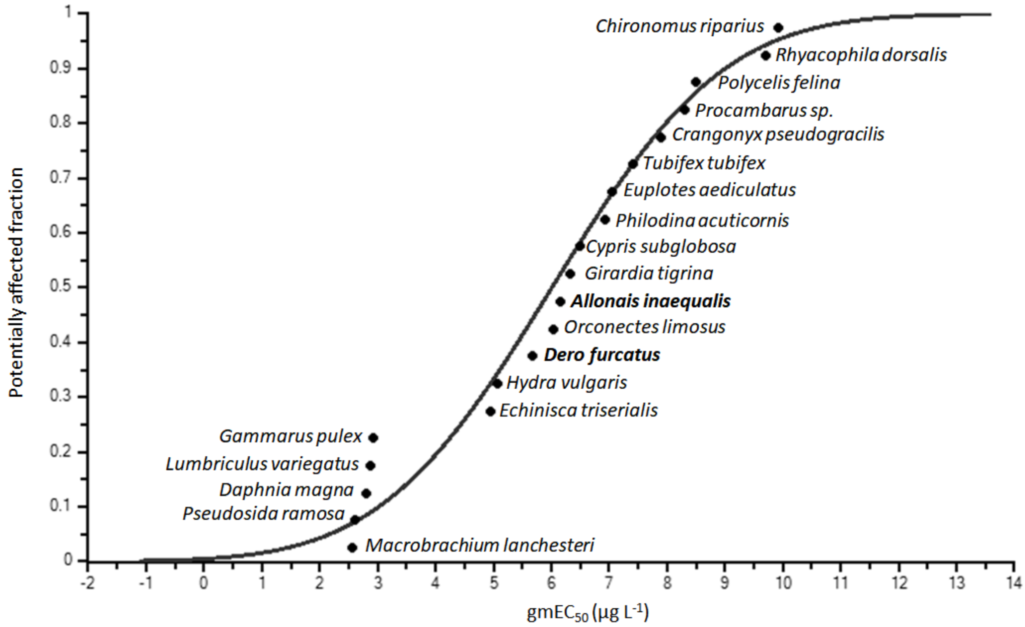


Figure 2. Range of sensitivity of *Dero furcatus* to copper sulfate based on the results of 20 acute toxicity tests. The upper and lower limits (95 % confidence intervals) were 0.0378 and 0.025 mg L^{-1} of copper sulfate.

3.2 Acute toxicity of single exposure to cadmium and mercury

Regarding the acute toxicity tests for both organisms, the mean values of 96-h LC_{50} obtained for cadmium and mercury and their respective standard deviation are shown in Table 2. Mercury toxicity to both oligochaetes was higher than that of cadmium chloride and *D. furcatus* was noted to be more sensitive than *A. inaequalis* to both metals (Table 2). In addition, both test species were found to be equally or more sensitive to the metals as compared to other frequently tested annelids in temperate regions such as *Tubifex tubifex* and *Lumbriculus variegatus* (Figure 1). Metal tolerances in aquatic oligochaetes have indeed been noted to be species-specific (Chapman et al., 1982). Large intra-species variation has also been demonstrated, as metal tolerance appears to depend on both acclimation and adaptation so that the organism source (from metal-polluted or uncontaminated sites) may largely impact their sensitivity to metals (Vidal and Horne, 2003). In addition, experimental conditions such as temperature and pH are also known to influence metal toxicity (Kim et al., 2017 and references therein). Chapman et al. (1982) demonstrated that cadmium toxicity was greater at greater temperatures and pH = 7 when compared to lower temperatures and pH 6 and 8.

A



B

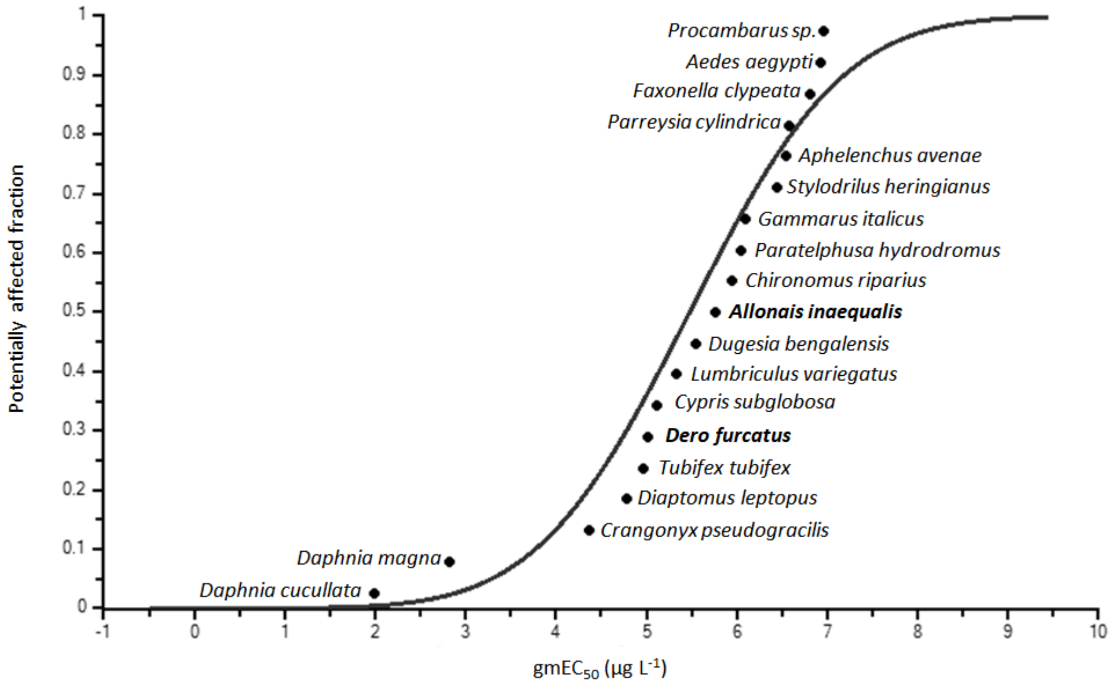


Figure 3. Species sensitivity distributions (SSD) constructed based on 96-h LC_{50} values (geometric means) for cadmium chloride (A) and mercury chloride (B) obtained in the present study for *Dero furcatus* and *Allonais inaequalis* (in bold) and from literature for other invertebrates.

Table 3. Mean values of hazardous concentration for 5% (HC_5) and 50% (HC_{50}) of the invertebrate community and their 95% confidence intervals (95% CI) for cadmium chloride and mercury chloride, based on SSD curves constructed from ecotoxicological data EC_{50}.

	HC_5		**HC_{50}**	
	HC_5 (μg L^{-1})	CI 95% (μg L^{-1})	HC_{50} (μg L^{-1})	CI 95% (μg L^{-1})
Cadmium chloride	127.317	2.5 – 1839.2	1018276.0	127309.9-8144615.0
Mercury chloride	1772.2	177.2 – 8433.9	300210.7	88574.8 – 1017518.0

3.3. Metal mixtures toxicity

Chemical mixtures with similar modes of action have traditionally been considered to act through concentration addition (CA), whereas the independent action (IA) model is used for chemical mixtures with dissimilar modes of action (e.g. Jonker et al. 2005; Loureiro et al. 2010; Moreira et al. 2017). A review by Wu et al. (2016) indicated that mercury reacts with nucleophiles of biomolecules during which oxidative stress may occur and results in the depletion of antioxidants of the biomolecules and an increase in the formation of reactive oxygen species (ROS), while cadmium indirectly generates ROS by its ability to replace iron and copper. Although the specific toxic pathway is hence slightly different, both eventually lead to the formation of ROS. Several studies have indicated that the concept of CA should be extended to mixture constituents having common apical endpoints or common adverse outcomes (Barata et al. 2012) such as is the case in the present study. In addition, the CA model has been recommended to be used as the standard toxicity prediction tool in ecological risk assessments since the endpoints are broader in terms of organizational level, and since they focus on individual survival, reproduction output, or growth (Damasceno et al. 2017; EFSA 2014). Although the CA model is generally more conservative than the IA model, the magnitude of the differences at low levels of exposure between the two models is usually small and hence, the outcome will not be overly conservative (EFSA 2013; Nørgaard and Cedergreen 2010).

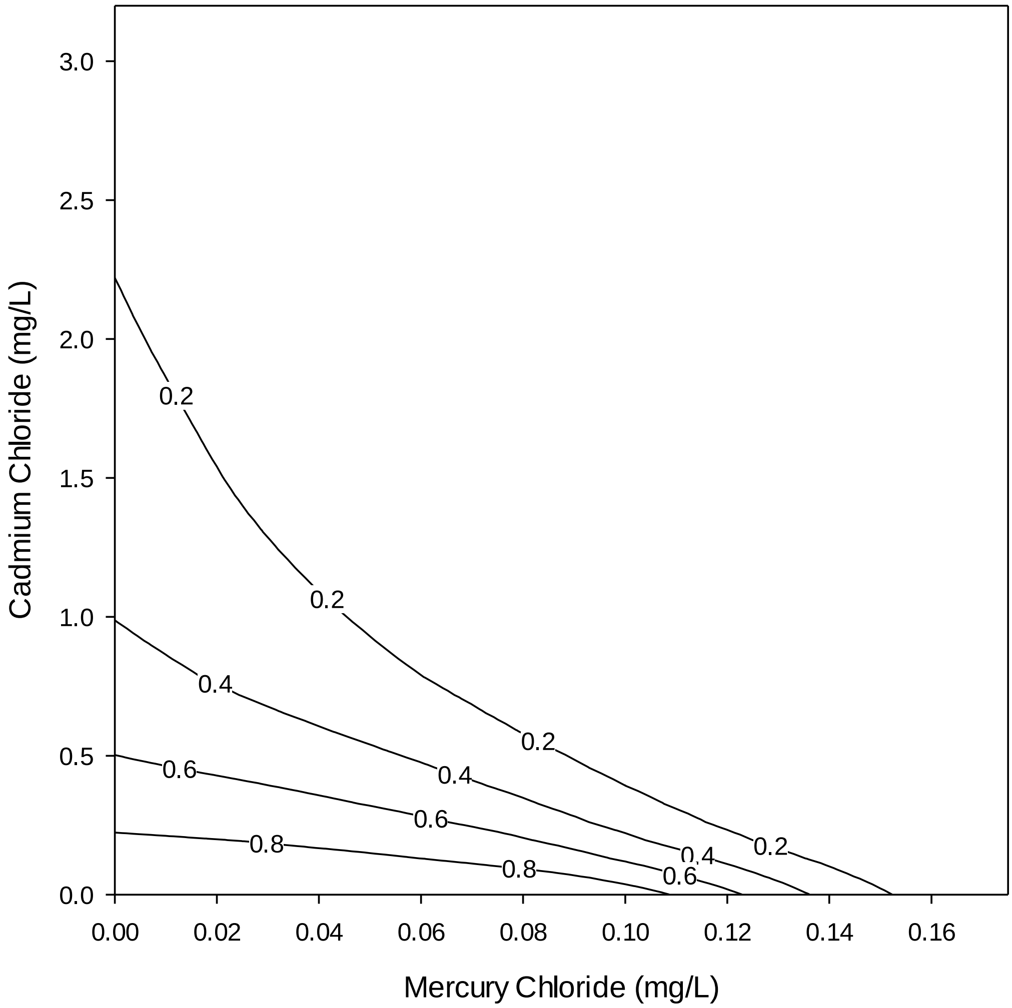


Figure 4. Isobologram of the effects of the metals mixtures on the survival of *Allonais inaequalis*, demonstrating a dose level-dependent (DL) deviation from the concentration addition (CA) model. The linear, concave and convex isoboles represent, respectively, no interaction, synergy and antagonism (Ryall and Tan, 2015).

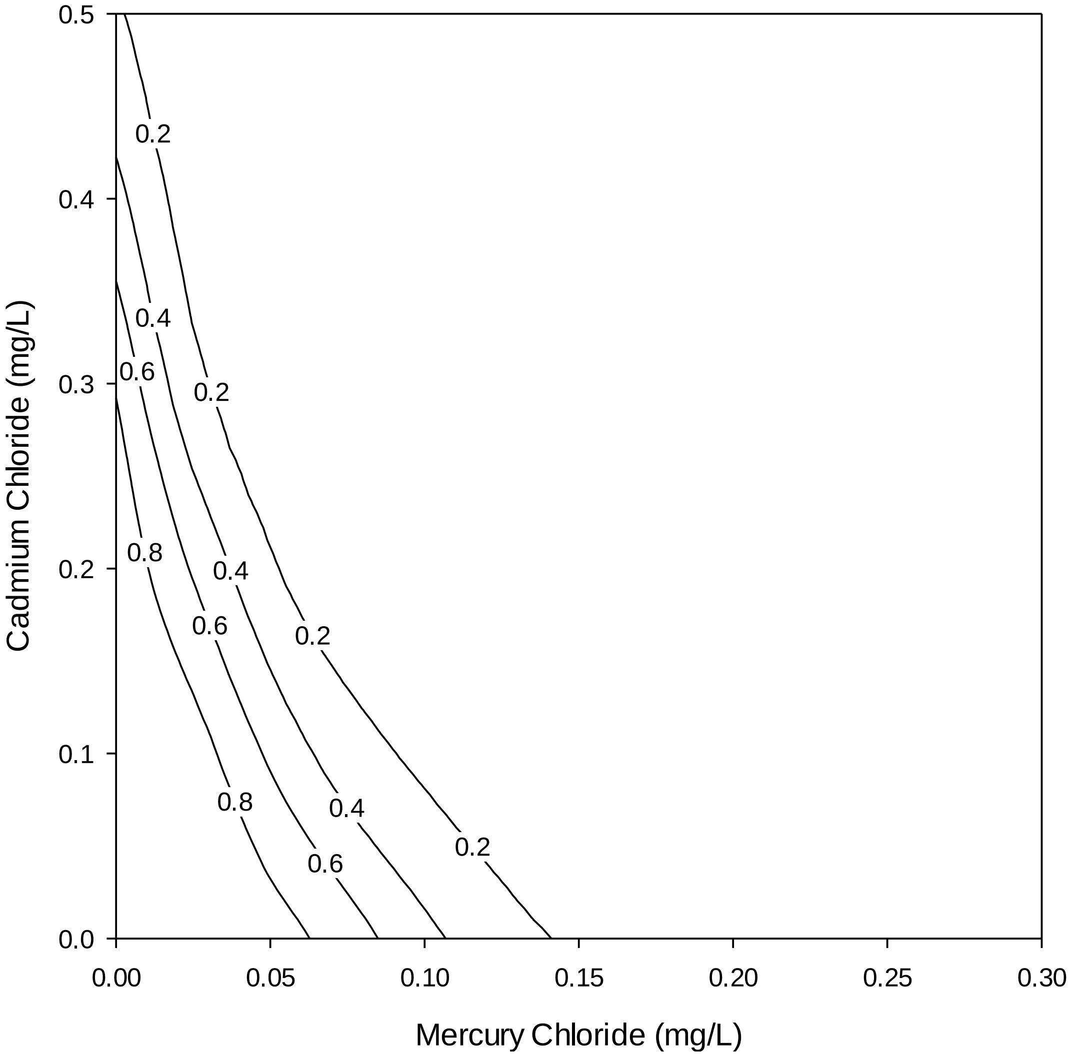


Figure 5. Isobologram of the effects of the metals mixtures on the survival of *Dero furcatus*, demonstrating a dose level-dependent (DL) deviation from the concentration addition (CA) model. The linear, concave and convex isoboles represent, respectively, no interaction, synergy and antagonism (Ryall and Tan, 2015).

In analyzing the results from the MIXTOOL analysis, the CA model generally better described the results of the data (sum of the squares of the residuals (SS) 38 and 33 for A. inaequalis and *D. furcatus*, respectively) although differences with the IA were minor. Subsequently, the results of the CA model analysis and deviations encountered for this reference model are provided in Tables 4 and 5 and visualized in Figures 4 and 5 for species *A. inaequalis* and *D. furcatus*. Based on the criteria set by Jonker (2005).

After the addition of the parameters "a" and "b" to the CA model, a dose level dependent deviation of the CA model was found to best describe the data for *A.*

inaequalis, as can be deducted from the significant decrease in the SS value to 23.17 (p < 0.05; r^2 = 0.94; Table 4). Therewith, the results of the mixtures showed that antagonism occurred at low and synergism at high concentrations of these metals mixtures. In addition, the results indicated that the change of the interaction occurred at the 96h-LC_{50} level (Table 4; Figure 4). For *D. furcatus*, after adding the parameter "a" to the model in order to describe the S/A deviation, the SS value decreased to 24.0 and was statistically significant (p < 0,05, r^2 = 0.95; Table 5). DR and DL deviations were not significant. Therefore, the S/A deviation from the CA model was the best fit for the data and indicated synergistic interactions of these metal mixtures (table 5; Figure 5).

Both antagonistic and synergistic toxic interactions between metals have been documented (see Wu et al., 2016 for a recent review). According to Cedergreen (2014), synergistic metal-metal interactions seem to be rare, although this appears to depend on the metal form, concentrations and organism tested. In line with the results of the present study, Mohan et al. (1986) noted a more-than-additive (synergism) interaction between cadmium and mercury in producing mortality of the intertidal bivalve *Perna viridis*.

Table 4. Summary of the analysis of acute toxicity of mixtures of mercury chloride and cadmium chloride for *Allonais inaequalis*.

	CA	S/A	DR	DL
max	0.93	0.91	0.91	0.98
β mercury chloride	13.63	13.95	13.97	8.67
β cadmium chloride	2.11	1.98	2.02	1.23
EC_{50} to mercury chloride	0.13	0.14	0.14	0.13
EC_{50} to cadmium chloride	0.68	0.80	0.80	0.70
a	-	- 0.47	- 0.31	2.30
$b_{DR/DL}$	-	-	- 0.23	1.00
SS	37.88	35.28	35.25	23.17
r^2	0.91	0.92	0.92	0.94
χ^2 or test F	391.95	2.60	0.02	12.10
df	-	1.00	1.00	1.00
p (χ^2/ F)	1.53×10^{-83}	0.10	0.88	0.0005

max = maximum value of the response; β = slope the individual response dose curve; LC_{50} = median effective concentration; a, b_{DR} e b_{DL} =s function parameters; SS = sum of the squares of the residuals; r^2 = regression coefficient; Test χ^2 or F = statistical test; df = degree of freedom; p (χ^2 / F) = level of significance for the statistical test. IA + independent action model, S/A = deviation synergism/antagonism, DR = dose ratio-dependent deviation and DL = dose level dependent deviation.

Table 5. Summary of the analysis of the acute toxicity test with mixtures of mercury chloride and cadmium chloride for the oligochaete *Dero furcatus*.

	CA	S/A	DR	DL
max	0.98	0.98	0.98	0.98
β mercury chloride	3.71	3.90	3.83	3.34
β cadmium chloride	6.37	5.70	5.85	5.05
EC_{50} to mercury chloride	0.07	0.09	0.10	0.09
EC_{50} to cadmium chloride	0.36	0.38	0.38	0.38
a	-	- 0.95	- 0.73	0.07
$b_{DR/DL}$	-	-	- 0.48	14.84
SS	33.30	24.0	23.45	22.27
r^2	0.94	0.95	0.95	0.95
χ^2 or test F	498	9.70	0.14	1.33
df	-	1.00	1.00	1.00
p (χ^2/ F)	2.06×10^{-106}	0.001	0.70	0.25

max = maximum value of the response; β = slope the individual response dose curve; LC_{50} = median effective concentration; a, b_{DR} e b_{DL} =s function parameters; SS = sum of the squares of the residuals; r^2 = regression coefficient; Test χ^2 or F = statistical test; df = degree of freedom; p (χ^2 / F) = level of significance for the statistical test. IA + independent action model, S/A = deviation synergism/antagonism, DR = dose ratio-dependent deviation and DL = dose level dependent deviation.

On the other hand, Solomon (2008) stated that cadmium interacts antagonistically with mercury in aquatic organisms and other metals other than mercury thus protect aquatic organisms from mercury uptake and toxicity.

4. Conclusions

This study evidenced the great importance of using local oligochaete organisms in ecotoxicological studies. This study also showed that cadmium and mercury in mixture may cause greater toxicity (synergism) to the benthic species than when tested individually. Thus, prospective environmental risk assessments and environmental quality criteria based on individual exposures may not adequately protect aquatic ecosystems.

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Conflict of interest: The authors declare that they have no conflict of interest.

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5.2. Effects of copper and manganese metals, isolated and in mixture, on the tropical indigenous oligochaetes *Dero furcatus* and *Allonais inaequalis*

Highlights

- Mixture toxicity of two metals was evaluated for two tropical indigenous Oligochaeta.
- Copper sulfate toxicity to both oligochaete species was higher than that of manganese sulfate.
- *Allonais inaequalis* was more sensitive than *Dero furcatus for* copper sulphate
- *Dero furcatus* was more sensitive than *Allonais inaequalis* regarding manganese sulfate toxicity.
- Synergism occurred in metal mixtures.

Abstract

Bearing in mind the evident discrepancy in the use of temperate organisms in detriment of tropical species and the indispensability of increasing research with organisms not yet evaluated, in this work we tested the sensitivity of the tropical freshwater worms *Allonais inaequalis* and *Dero furcatus* (Oligochaeta, Naididae) to two metallic compounds: copper sulfate and manganese sulfate, both, isolately and in mixtures. The 96h LC_{50} of copper sulfate was 25.75 μg L^{-1} and for *A. inaequalis* and 31.39 μg L^{-1} for *D. furcatus.* For manganese sulfate, the LC_{50} was 28155.16 μg L^{-1} for *A. inaequalis* and 18250.62 μg L^{-1} for *D. furcatu*s, much higher than the LC_{50} values copper sulfate. Comparing these values it is evidenced that manganese has an acute toxicity almost one order of magnitude lower than copper to these benthic oligochaetes. Mixture combinations of these two metals showed synergism between copper sulfate and manganese sulfate, what shows that although the isolated manganese does not present high toxicity, its natural occurrence concomitantly with other metal can potentiate the toxic effects. The toxicity of both metals was compared to those already found for phylogenetically related species and also with other groups of invertebrates by plotting Species Sensitivity Distribution curves (SSDs). The curves showed that the sensitivities of both oligochaete species were more sensitive than other test-species frequently used in toxicity essays such as *Limnodrilus hoffmeisteri*, *Tubifex tubifex, Lumbriculus variegatus* and *Chironomus sp*. In conclusion we found that the sensitivity of *Allonais inaequalis* and *Dero furcatus* was high when compared to annelid species internationally used in ecotoxicity essays, which support the possibility of using tropical organisms in these trials, since they are more easily obtained and cultured. Preference on adoption of these species for ecotoxicity testing in tropical regions is an alternative that takes into account the heterogeneity of environmental conditions at different latitudes and that also reveals the existence of convergence in the evolutionary adaptation of species to tropical or temperate systems.

Keywords: Ecotoxicology; Metal mixtures; Annelida; Tropical species; Species Sensitivity Distribution.

1. Introduction

A metallic pollutant rarely exists isolated in the environment (Meyer et al., 2015), wherein the toxic degree of this contaminant can be attenuated or potentiated due to the interactions that occur with other substances, whether inorganic or organic, such as metals and pesticides. The interactions between the compounds can alter many physiological processes in organisms, such as assimilation, metabolism and excretion, which are usually related, on a cytological scale, to oxidation stress (Jaishankar et al., 2014), that leads to DNA damage, lipid peroxidation and protein modification through specifics sites binding (Jorge et al., 2016; Mansano et al., 2017; Wu et al., 2016).

Two compounds with similar modes of action in mixtures are expected to show additive toxicity effects, according to the concentration of the compounds; thus their combined effect is nothing more than the sum of their isolated effects (Verslycke et al., 2003). However, when the contaminants present some type of interaction the final result may be more-than-additive or less-than-additive than expected by the simple sum of their toxicity (Franklin et al., 2002). Although these mechanisms can be observed through ecotoxicity essays, there is still a lack of mechanistic understanding which makes it difficult to predict the effects of these mixtures on the aquatic ecosystem (Meyer et al., 2015), a gap with serious consequences since the prior detection of the toxicity of contaminants and their mixtures is vital in the elaboration of strategies to reduce and mitigate the deleterious effects of pollutants (Utgikar et al., 2004).

Since water has universal solvent properties, aquatic organisms are even more susceptible and exposed to the metal compounds. In addition to interactions between metal compounds, toxicity to aquatic organisms is enhanced by multiple factors (Balistrieri and Mebane, 2014) such as pH, temperature, water hardness, organic matter deposited on the substrate, and other factors that influence single and mixture metals toxicity. Another important factor in predicting whether a pollutant (Franklin et al., 2002; Yim et al., 2006) will cause harmful effects to an organism is the bioavailability of the contaminant, either in the water column or in the sediment (Santore and Ryan, 2015; Wu et al., 2016).

Sediments play an important role in the availability of metals in the aquatic environment according to the partitioning behavior or binding strength of the contaminant to the sediment (Wu et al., 2016) what has been extensively studied since they may represent a source of release of metallic compounds into the water column or a sink for their deposition, according to the oxidation potential or reduction of the studied environment (Audry et al., 2004; Hornberger et al., 1999).

Studies have reported that most metals in the water occur in non-bioavailable forms for organisms (Bervoets and Blust, 2003; Martins et al., 2015), a favorable point for species living in the water column but negative for those who live temporary (life stage or resource search) or permanently (benthic organisms) in the sediment, as in the case of the oligochaetes of the Naididae family (Smith et al., 1991). Among the consequences of high concentrations of metal compounds in the sediment and in the water column is their bioaccumulation in both, invertebrates and vertebrates (Jayaprakash et al., 2015; Rainbow, 2002), as a result from the discrepancy between assimilation and elimination rates, once these compounds are not destroyed in living organisms by biological degradation, thus accumulating in tissues with potential for being biomagnified in the trophic chain (Dengyue Yuan et al., 2017).

In aquatic ecosystems the base of the food chain, the autotrophic organisms, such as phytoplankton and macrophytes, has great potential for assimilation and bioaccumulation of metals (de Souza Lima Junior et al., 2002), which are transferred to the next trophic levels through feeding, from the primary consumers such as herbivorous zooplankton and fish to the top-chain predators, leading to an increase in metal concentrations at highest levels (Goodyear and McNeill, 1999; Voutsas et al., 2002). Contaminant transfer through consumption is not the only way to contaminants bioaccumulation. Sediments are a source of toxicity, since metals are incorporated into the sediment and since biota, especially benthic, is constantly exposed to it and to interstitial water (Corbi et al., 2010).

Although natural copper entry many processes occurring in the aquatic environment, such as soil erosion and geological weathering (Jayaprakash et al., 2015), the greatest entry of this metal into ecosystems occurs by anthropic action, through industrial, agricultural and domestic activities (Jaishankar et al., 2014; Jorge et al., 2016). Copper is an essential micronutrient, being extremely important in many enzymatic systems in trace amounts, acting like an enzyme co-factor (Franklin et al., 2002), but high concentrations of this metal can be extremely toxic, causing nephritis, anuria, and extensive kidney damage (Mansour and Sidky, 2002). One of its main sources is the use of copper-based compounds in the treatment of sewage and as fungicides in the agriculture (Knakievicz and Ferreira, 2008).

Manganese is widely found in surface soils, in most rocks and soil types, aquatic sediments, groundwater and surface waters with wide variation in their concentration, generally in inorganic form (Sköld et al., 2015) and play an important role in the physiological mechanisms of aquatic species, since it is an important co-enzyme factor (Harford et al., 2015; Jayaprakash et al., 2015). Due to its high occurrence in industrial processes, manganese emerges as a potential contaminant in high concentrations, mainly in the vicinity of metal mining areas (Andrade et al., 2010; Stubblefield et al., 1997). This metal is important in metabolic processes, bone mineralization and citological protection, although discrepant levels in its presence can lead to diseases such as manganism, which resembles Parkinson's disease (Sköld et al., 2015).

The objective of this work was to evaluate the toxicity of copper sulfate and manganese sulfate in an isolated manner and in mixtures to verify the occurrence of interaction between these metals when in contact with aquatic biota. It also intended to compare the isolated acute toxicity results obtained for the tested Oligochaeta species (*Dero furcatus* and *Allonais inaequalis*) with other aquatic invertebrate species.

2. Materials and Methods

2.1. Test organism and culture conditions

Dero furcatus individuals were collected from aquaculture tanks belonging to the Department of Hydrobiology located at the campus of UFSCar (São Carlos, Brazil - 21°58'54.7"S 47°52'39.3"W) and kept for at least one week in the Ecotoxicology Laboratory at the Department of Ecology and Evolutionary Biology (DEBE) in the same University for acclimation, according to the established standards by the Bioaccumulation in Sediment-dwelling Benthic Oligochaetes protocol (OECD, 2008). The same guide protocol was used in the acclimatization and maintenance of the individuals of *Allonais inaequalis* Stephenson, 1911 (Oligochaeta: Naididae) obtained in a culture preset for more than 24 months in the Hydraulics and Sanitation Department of the University of São Paulo, Brazil) and also acclimatized in DEBE. The ranges determined by the protocol were as follows: 22°C ± 2.5°C temperature; 16 h light : 8 h dark photoperiod; 90.0 - 400.0 mg CaCO3 L^{-1} hardness; 6.0 - 9.0 pH; besides 410 – 500 μScm^{-1} conductivity, that were followed and checked; all these parameters were measured weekly. Twenty (20) mL of Tetramim® (5g in 2L of distilled water) - were used weekly for the oligochaetes feeding.

2.2. Chemicals and test solutions

Stock solutions were prepared at concentrations of 10mg L^{-1} and 100mg L^{-1} for copper sulfate ($CuSO_4$ - CAS no: 7758-98-7) and manganese sulfate ($MnSO_4$ - CAS no: 7785-87-7), respectively, prepared immediately before the use in experiments. The nominal concentrations were calculated according to the dilution of the stock solution in the culture medium used in the cultures. The preliminary and definitive acute tests followed the protocols stipulated by OECD (2008).

The sensitivity tests carried out for attesting the health of the organisms and, thus, validating the acute final tests, were carried out monthly, in a total of 20 tests, with the reference substance copper sulfate ($CuSO_4$). During the tests the water parameters such as hardness, conductivity, pH and temperature were measured both at the beginning and at the end of the tests.

2.3. Acute toxicity tests: isolated compounds and their mixtures

The acute toxicity (96h) tests with copper sulfate and manganese sulfate followed the parameters established for benthic invertebrates (OECD, 2008), of which: four replicates for each treatment, six organisms per replicate and five concentrations for each test compound, in addition to control. In order to verify the reproducibility of the tests and to predict the possible variations in the results, five tests were performed for each of the metal compounds.

Six adults were used for the isolated tests and five for the mixtures tests, according to the availability of organisms; they were placed in plastic cups with 10 mL of the test solution or the reconstituted water for control; the average size for *A. inaequalis* was 7.8 mm and for *D. furcatus* 7.0 mm.

The acute tests had their concentration ranges established through preliminary tests, in which several concentrations were tested. For copper sulfate the definitive concentration range adopted for both oligochaetes was: 0.005; 0, .01; 0.02; 0.04; 0.06 and 0.08 mgL^{-1}, while the concentration range of manganese sulfate was 17,5; 18,4; 19.4; 20.4; 21.4 e 22.4 mgL^{-1} for *D. furcatus* and 27.5; 28.4; 29.4; 30.4; 31.4 e 32.4 mgL^{-1} for *A. inaequalis.*

All acute ecotoxicity assays were maintained in the dark, without feed and at controlled mean temperature of 22 ± 2.0°C, with readings in 48h and 96h. Such readings were performed through observations of the organisms under stereomicroscope: all dead organisms were recorded and used for the lethal concentration calculation (96-h LC_{50}).

The evaluation of the combined effects between copper sulfate and manganese sulfate was performed by exposing the organisms to both the isolated metals and their combination simultaneously, through a binary toxicity factorial design containing 25 combinations. Exposures with the combinations were assembled by the same methods as described for acute toxicity tests.

2.4. Data analysis

For the acute toxicity tests 96-h LC_{50} values (95% confidence intervals) were calculated by PriProbit free software. Difference was considered significant when $p \leq 0.05$, in all statistical tests.

2.5. Species sensitivity distribution

In order to compare the acute LC_{50} values of the studied Oligochaeta species (*D. furcatus* and *A. inaequalis*) with the values of other invertebrate species present in the literature, Species Sensitivity Distributions (SSDs) curves were constructed with data from copper sulfate and manganese sulfate results. Ecotoxicity data were compiled from the USEPA ECOTOX database (http://cfpub.epa.gov/ecotox/, 2018) with the aid of free available literature. When more than one value was found for the same species the geometric mean of these values was calculated. Through the program ETX computer program, version 2.0 (Van Vlaardingen et al., 2004) log-normal distributions of threshold values were constructed. Both, the hazardous concentration for 5% of the species (5th percentile – HC5) and the hazardous concentration for 50% of the species (50th percentile - HC50) with their confidence limits were calculated with the software based on the methodology described by Aldenberg and Jaworska (2000). Log-normality was tested with the Anderson-Darling test included in the ETX software package since the model assumes a log-normal distribution of the data, which was evaluated at the 5% significance level.

3. Results and Discussion

During the acute tests and mixtures for both Oligochaeta (*D. furcatus* and *A. inaequalis*) the test solutions were kept within the following ranges: conductivity from 415.6 to 456.8 $\mu S cm^{-1}$; hardness from 174 to 202 mg $CaCO_3$ L^{-1}; pH from 7.1 to 7.6 not varying by more than 1.0 unit; and temperature from 22.6 to 24.5ºC. All physical and chemical variables met all the validity criteria laid down by the Bioaccumulation in Sediment-dwelling Benthic Oligochaetes protocol (OECD, 2008).

3.1 Acute toxicity

The mean value (96-h LC_{50}) of the reference substance copper sulfate ($CuSO_4$) and its 95% confidence limits for the oligochaetes *D. furcatus* and *A. inaequalis* were 0.0314 (0.025 – 0.0378) mg L^{-1} and 0.02575 (0.0234 – 0.0281) mg L^{-1}, as already reported in Chapter 1.

Table 1. Mean values of LC_{50} and standard deviation (SD) obtained in acute toxicity tests of two different metals to the oligochaete species *Dero furcatus* and *Allonais inaequalis*.

Compounds tested	***Dero furcatus*** **96h LC_{50} (μg L^{-1})**	***Allonais inaequalis*** **96h LC_{50} (μg L^{-1})**
Copper Sulfate	31.39 ± 3.21	25.75 ± 1.15
Manganese Sulfate	18250.62 ± 61.74	28155.16 ± 63.08

All survival rates observed at controls at the end of acute tests and mixtures were higher than 95%. In the acute toxicity tests evaluating the individual metal compounds, LC_{50}-96h values for copper sulfate and manganese sulfate were calculated separately for each of the five tests. The mean values of the 96-h LC_{50} and their respective standard deviations (mean ± SD) for *D. furcatus* were 31.39 ± 3.21 μg L^{-1} for copper sulfate and 18250.62 ± 61.74 μg L^{-1} for manganese sulfate, and, for *A. inaequalis* were 25.75 ± 1.15 μg L^{-1} for copper sulfate and 28155.16 ± 63.08 μg L^{-1} for manganese sulfate; all tests were based on nominal test concentrations as showed in Table 1.

Copper sulfate toxicity level was 581 times greater than manganese sulfate when tested for *D. furcatus* and 1093 times greater when tested with *A. inaequalis*. In general, toxicity is a consequence of non-specific binding of reactive metal cations with essential macromolecules to the organism functioning, which causes changes in the mode of action of these molecules. In aquatic invertebrates, metal ions such as manganese are accumulated in large quantities on the body surface and bind to the cuticle, with reduced impact on internal mechanisms of homeostasis, whereas copper accumulates in cells, especially in the cytosol, causing more severe damage to organism. Copper penetrates through the cell membranes of invertebrate organisms by active transport and has the potential to morphologically and physiologically modify parameters such as growth rate, feed consumption, respiration rate, production, survival, and the life cycle through non-specific intracellular binding (Golovanova, 2008). Although with minor toxicity, as reported by Stubblefield et al. (1997), studies regarding manganese toxicity for Oligochaeta (*Eisenia fetida* and *Enchytraeus crypticus*) have already been carried out by other authors due to their ubiquitous occurrence in soil and the direct contact that these invertebrates have with it (Kuperman et al., 2004; Sköld et al., 2015). The high toxicity of copper has already been reported by Howe et al (2014) who demonstrated the high toxicity of copper among metals such as cadmium, nickel and zinc in tests performed with the anemone *Aiptasia pulchella*. In high concentrations copper even inhibits algal growth as shown by Franklin (2002), interfering in processes such as photosynthesis, respiration, adenosine triphosphate (ATP) production, and pigment synthesis. It was also possible to verify that *Dero furcatus* was more sensitive to manganese sulfate than *Allonais inaequalis*, while the last was more sensitive to copper.

To verify the toxicity of the mixtures of copper sulfate and manganese sulfate, an analysis was made of the percentages of mortality for the concentrations of isolated metals concomitantly with the solutions containing both of them (Table 2). Mixtures of metal compounds tend to interact with active sites of organisms, thereby displacing original metals from their natural bonding points (Jaishankar et al., 2014). Some effects are basic when dealing with individual metals whose toxicity affects physiological processes such as the absorption of food or water by the body, as demonstrated by Faulk et al. (2014) and Mousa et al. (2014), who reported the change in the consumption of food and water from different organs in exposure to metallic toxic substances. Metal mixtures can lead to more serious effects due to the synergistic processes that metals can present when their modes of action are similar or different but with the potential to damage the same physiological process. One of the effects studied by Wu et al. (2016) is the effect of mixtures on the permeability of the plasmatic membrane, since damaged biological membranes allow the entry of toxic chemical components that can lead to malfunction or even cell cytolysis. Another problem posed by the toxic potential of mixtures is the decrease in the intracellular concentration caused by the decrease in the number of cells, with direct consequence in the toxicity that the organism will experience (Wu et al., 2016).

For the mixture between copper and cadmium, Franklin (2002) reports that the toxicity may have been a result of the alteration of the cell membrane and the effects of both metals on their permeability. Finally, another serious effect recorded by the interaction between metals in the cell nucleus is the binding of metals to DNA and nuclear proteins due to the oxidative deterioration of biological macromolecules (Jaishankar et al., 2014). The effects however, may not always present additive or synergistic results: the mixtures of Cd, Cu, Zn, Pb on *Daphnia magna* showed toxicity that did not differ from the sum of their effects despite the different hardness of the tested water (Yim et al., 2006).

Table 2. Immobility (in %) in the acute laboratory tests evaluating the acute toxicity of mixtures of copper sulfate and manganese sulfate for the oligochaetes *Dero furcatus* (A) and *Allonais inaequalis* (B)

A

	Manganese Sulfate (mg/L)						
Copper sulfate (mg/L)	**0**	**16.5**	**17.5**	**18.4**	**19.4**	**20.4**	**21.4**
0	0	0	30	45	90	100	100
0.01	0	35	75	85	100	100	100
0.02	5	75	90	100	100	100	100
0.03	45	85	95	100	100	100	100
0.04	85	100	100	100	100	100	100
0.05	95	100	100	100	100	100	100
0.06	100	100	100	100	100	100	100

B

	Manganese Sulfate (mg/L)						
Copper sulfate (mg/L)	**0**	**26.5**	**27.5**	**28.4**	**29.4**	**30.4**	**31.4**
0	0	0	25	50	95	100	100
0.01	0	40	70	90	100	100	100
0.02	30	70	85	95	100	100	100
0.03	60	90	95	100	100	100	100
0.04	80	100	100	100	100	100	100
0.05	95	100	100	100	100	100	100
0.06	100	100	100	100	100	100	100

The main effect reported for freshwater invertebrates exposed to copper is the deregulation in the monovalent Na^+ cation; the metal eventually reduces absorption of ion uptake, which leads to an imbalance in the level of sodium circulating in the body, an effect possibly associated with inhibition caused by copper in the activities of the enzymes associated with the osmoregulation process, such as Na^+, K^+ -ATPase and carbonic anhydrase (Jorge et al., 2016).

3.2. Species sensitivity distributions

Data for the HC_5 and HC_{50} values are given in the supplementary material (Table 3) together with their respective confidence intervals, constructed from the compilation of the acute toxicity data for copper sulfate and manganese sulfate from databases.

Table 3. Mean values of hazardous concentration for 5% (HC_5) and 50% (HC_{50}) of the invertebrate community and their 95% confidence intervals (95% CI) for copper sulfate and manganese sulfate, based on SSD curves constructed from LC_{50} ecotoxicological data.

	HC_5		**HC_{50}**	
	HC_5 (μg L^{-1})	CI 95% (μg L^{-1})	HC_{50} (μg L^{-1})	CI 95% (μg L^{-1})
Copper sulfate	8,647	(2,536 - 20,034)	146,196	(75,998 - 281,235)
Manganese sulfate	93,400	(4,354 - 589,985)	13307,050	(2909,195 - 60868,250)

Analysis of the SSD curves allows us to infer that, for copper sulfate (Figure 1A), both tropical indigenous species of Oligochaeta showed high sensitivity to the studied compound when compared with other aquatic invertebrates, with LC_{50} values close to those daphnids of recurring use in ecotoxicity essays, such as *Ceriodaphia dubia*. Also, they were highly sensitive when compared to standardized temperate annelids, such as *Lumbriculus variegatus, Limnodrilus hoffmeisteri* and *Tubifex tubifex.* In relation to manganese sulfate (Figure 1B), *D. furcatus* and *A. inaequalis* presented intermediate sensitivity, smaller than those of *Daphnia obtuse* and *Chironomus javanus*, but superior to that of *Tubifex tubifex*.

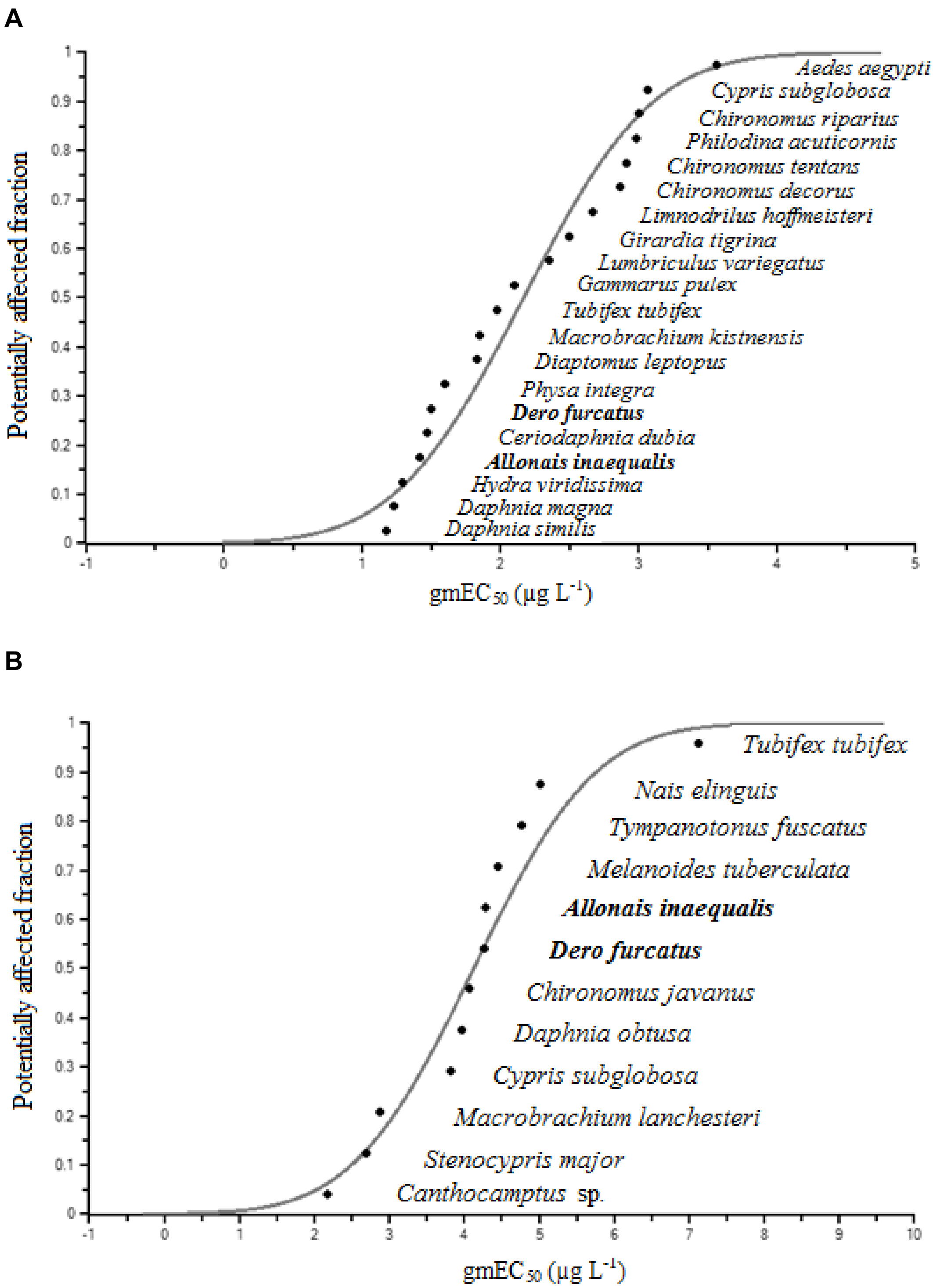

Figure 1. Species sensitivity distributions (SSDs) constructed based on 96-h LC_{50} values (geometric means) for copper sulfate (A) and manganese sulfate (B) obtained in the present study for the oligochaetes *Dero furcatus* and *Allonais inaequalis* (in bold) and from literature for other invertebrates.

Many other studies have compared tropical organisms with their counterparts used in temperate countries, for different groups (Buch et al., 2017; Daam and Rico, 2016; Ghose et al., 2014; Pelli L Howe et al., 2014; Pelli L. Howe et al., 2014; Mansano et al., 2016; Moreira et al., 2017; Silvano and Begossi, 2016). It is therefore clear the importance of collecting data that support and validate the ecotoxicological studies for tropical species, such as that of Silvano and Begossi (2016), who studied patterns of bioaccumulation of mercury in fish muscles of 69 tropical and subtropical fish species in relation to their trophic levels, or of Ghose et al. (2014), who performing tests with tropical amphibians and pesticides recurrently used in Costa Rica and indicated a direct relationship between the lack of ecotoxicological knowledge on these pesticides for tropical species and the mass extinctions of amphibians across the globe. However, this comparison between tropical and temperate species has to take into account the differences between physics, chemical and biological parameters (e.g., pH, dissolved oxygen, temperature, pH, hardness, conductivity, photoperiod), which may influence the levels of toxic effects of pollutants or even the mortality of organisms tested (Moreira et al., 2014).

4. Conclusions

The present work evidenced the importance of the use of benthic organisms in ecotoxicological studies, as observed by SSD comparisons that indicate that both tropical indigenous oligochaetes species tested are sufficiently sensitive and therefore recommended as a test organism in toxicity tests. Although copper is notably more toxic than manganese to benthonic oligochaetes, this work has shown that in mixtures the latter metal potentiates the effects of the former, making both equally hazardous to benthic aquatic biota. Thus, it is argued that the environmental toxicity criteria isolated for both metals may not account the actual risk they present when occurring concomitantly.

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5.3. Chronic effects of pesticides and metals on the tropical Oligochaeta species *Allonais inaequalis*

Highlights

- Chronic toxicity of four metals and two pesticides was evaluated to a tropical indigenous Oligochaeta.
- Copper sulfate treatment caused a significant decrease in relation to survival and growth.
- Mercury and Cadmium chloride treatments showed no significant differences in mortality data although it showed significant decrease in the organisms' growth.
- Carbofuran caused a significant decrease in relation to survival and growth.
- Diuron caused no statistical difference in relation to mortality and growth.

Abstract

Given the growing need to find species that reflect local long-term problems, there is an urgent need of using native organisms that are evolutionary adapted to large scale different environmental conditions, such as those existent in the polar, temperate and tropical regions, and with high sensitivity, so that they can reflect and act as indicators of ongoing impacts whether from punctual or diffuse sources. Based on these questions, the objective of this study was to evaluate the feasibility of using the oligochaete *Allonais inaequalis* as test-species for 12-day chronic toxicity essays with the metal compounds copper sulfate, manganese sulfate, mercury chloride and cadmium chloride, and with the pesticides carbofuran and diuron, in their commercial formulations. In relation to organisms survival rate, a sharp drop was observed after the long-term exposure to the metal copper sulfate and the commercial formulation of the pesticide carbofuran. On the other hand, there were no statistical differences in the survival rate at the end of the twelfth day of chronic toxicity experiment with manganese sulfate and the herbicide diuron. The results regarding growth rates

were similar, re-inforcing the high toxicity of the first four compounds in contrast to the low chronic toxicity of manganese and diuron. The present results demonstrated the deleterious effect that chronic (sub-lethal) exposure to the studied substances can cause to benthic organisms, which are extensively exposed to contaminants that can be particularly accumulated in sediments where they thrive and which have great potential for bioaccumulation. Changes in life cycle important aspects such as growth can be harmful to sexual maturity and reproductive capacity of a population, as they can lead to a population bottleneck and its consequent extinction.

Keywords: Ecotoxicology; Metal; Pesticides; Annelida; Long-term effects; Tropical species.

1. Introduction

Metals can enter into aquatic ecosystem in innumerable ways, either by natural or anthropogenic activities (Ayanka et al., 2017). Even in small quantities, metals can accumulate in aquatic organisms according to its speciation in the sediment or dispersed in the water column. In addition, some of them may bioaccumulate along the trophic chain as they are ingested along with the food present in the sediment or assimilated by direct body contact with water, causing damage to cell membranes, enzymatic complexes as well as damage to the DNA (Buch et al., 2017). The metals bioavailability and toxic potential in freshwaters is greatly influenced by water parameters such as pH and dissolved oxygen (Joachim et al., 2017). Cadmium is a non-essential metal for the organisms' physiological mechanisms with high environmental persistence and widely distributed in aquatic environments. It enters ecosystems by anthropogenic and natural means, since it is used as a component of smelting, battery manufacture, paints, fertilizers and an atmospheric fallout from fossil fuels combustion, that can be transported by atmosphere bound to fine particles (Burger, 2008; Dolagaratz Carricavur et al., 2018). Mercury is a liquid metal at ambient temperature and pressure which is very soluble in water and thereby become very bioavailable and toxic, especially in the form of salts (Boening, 2000). In addition to its high persistence and a high bioaccumulation factor, mercury is widely distributed in its atmospheric form, since between 1010 - 4070 tons of mercury are emitted per year with a residence time of approximately one year, which makes it ideal to travel great distances and be deposited in the most diverse environments (Buch et al., 2017).

Copper is a particularly hazardous metal because of its widespread occurrence in industrial and agricultural dumps and its efficiency as fungicide, bactericide, plant herbicide, molluscicide, and algicide, and may cause internal hydromineral regulatory functions to be overwhelmed, damaging physiological processes and even killing organisms (Joachim et al., 2017). Although laboratory results indicate that manganese has a reduced environmental impact when compared to other metals, and its acute and chronic toxicity is generally low for biota (Harford et al., 2015), its toxicity can be increased by pH, redox potential, dissolved oxygen and organic matter (Sanders et al., 1998) since this metal is an essential antioxidant enzymatic factor of several biochemical reactions, necessary for the metabolism of carbohydrates, lipids, aminoacids and proteins and since manganese is an abundant element on Earth (Kuperman et al., 2004; Martin et al., 2008; Michalke and Fernsebner, 2014). It is widely used as a fertilizer base and in the production of steel and batteries (Harford et al., 2015).

Pesticides are substances commonly used in agricultural practices, despite their known effects on non-target organisms, with serious consequences for food chains and ecosystem health. Although accidental spraying presents a relatively small risk to aquatic biota, leaching and runoff water present a more serious problem due to the large amounts of contaminants being hauled (Dobsikova, 2003). Diuron [(N-(3,4-diclorophenyl)-N,N-dimethylurea] is a herbicide of moderate solubility with high environmental persistence, which produces high mortality rates in high concentrations. Although these concentrations are not always representative of natural conditions, sub-lethal concentrations that cover all or part of the animal's life cycle can severely alter components such as neurotransmitters, hormones, immune response, reproduction, physiology, morphology or behavior (López-Doval et al., 2014). Carbofuran is a systemic broad-spectrum insecticide, nematicide and acaricide, which has been widely used throughout the world and thus has been detected in a wide range of terrestrial and aquatic ecosystems (Dobsikova, 2003). This pesticide presents high solubility and low adsorption coefficient, which makes it present in surface runoff and with great potential of accumulation in water bodies (Jayatillake et al., 2011). It irreversibly binds to the enzyme acetyl cholinesterase, inhibiting its action on the metabolite acetylcholine (Moreira et al., 2015).

Due to their abundance and key position in the passage of matter and energy along the trophic chain, especially in lotic systems, invertebrates play an essential role in the study of the contaminants flow and bioaccumulation (Joachim et al., 2017). Among invertebrates, Oligochaeta are excellent environmental indicators due to their sensibility regarding their mucous epidermis high absorption capacity and because these organisms have colonized a large number of aquatic and semi-aquatic ecosystems, freshwater or marine water. Its detritivorous habit is a very useful tool for the detection of pollutants in sediments and organic matter (Kusturica et al., 2015). Sediment acts as a sink or source of metal contaminants and pesticides, which makes benthic organisms direct target from these pollutants and susceptible to physiological stress (Dolagaratz Carricavur et al., 2018). Thus, benthic macroinvertebrates, and among them Oligochaeta, is one of the main groups used to monitor, evaluate and quantify impacts on aquatic environments, since they spend part of their life in the sediment and part in the water column, an instrument to measure contaminant damage in both compartments (Baumart et al., 2011).

Since organisms are usually exposed in a chronic way to low concentrations of metals and pesticides present in water and sediment, tests that study the animal's life cycle tend to be more realistic than acute tests with high concentrations of the contaminants (Pasteris et al., 2003). Thus, the present study carried out chronic essays with the Oligochaeta *Allonais inaequalis* for copper sulfate, manganese sulfate, cadmium chloride, mercury chloride and for the commercial formulas of the pesticides carbofuran and diuron, in order to verify the long term lethality and the influence of the compounds on the animal growth rate.

2. Materials and Methods

2.1. Test organism and culture conditions

Allonais inaequalis Stephenson, 1911 individuals (Oligochaeta: Naididae) was obtained at the Hydraulics and Sanitation Department of the University of São Paulo (São Carlos, Brazil) from stock cultures maintained for more than 24 months and cultivated in the Department of Ecology and Evolutionary Biology, Federal University of São Carlos (UFSCar - 21°58'54.7"S 47°52'39.3"W - São Carlos, Brazil), at the Ecotoxicology Laboratory. Acclimatization occurred for at least one week before the toxicity tests. During this period, oligochaetes were kept in 10 L aquaria filled with reconstituted water with the following stipulated conditions (OECD, 2008): temperature (22 ± 2.5°C) measured with Oximeter Hanna, USA, model HI9146; photoperiod of 16h light : 8h dark; conductivity between 410 and 500 $\mu S cm^{-1}$ measured with Digimed Conductivity meter (DO-48 model, Brazil); hardness between 90.0 and 400.0 mg $CaCO_3$ L^{-1} calculated as total calcium carbonate by titration method (Mackereth et al. 1978); pH between 6.0 and 9.0 measured with Micronal pH meter (model B371, Germany). Oligochaetes were fed once a week with 20 mL Tetramim® fish feed solution (5 g Tetramim® to two liters of distilled water).

2.2. Chemicals and test solutions

Stock solutions of copper sulfate (10 mg L^{-1} - CuSO4 - CAS no: 7758-98-7), manganese sulfate (100 mg L^{-1} - MnSO4 - CAS no: 7785-87-7), cadmium chloride (10 mg L^{-1} - $CdCl_2$ – CAS no: 10108-64-2 – Carlo Erba) and mercury chloride (10 mg L^{-1} - $HgCl_2$ – CAS no: 7487-94-7 – ACS Merck). Carbofuran and Diuron commercial formulations were used for the tests: Furadan® 350SC (purchased from FMC, Brazil - 35% m/v active ingredient and 65% m/v inert ingredients) and Diuron Nortox® 500 SC (Nortox S/A, Brazil - 50% m/v active ingredient and 69.4% m/v inert ingredients), respectively. Both, pesticides and metals stock solutions were prepared immediately prior to the toxicity tests. For calculation of test substances nominal concentrations, dilution was performed with the stock solution in culture medium.

Through protocol issued by OECD (2008), preliminary tests were performed to determine the experimental concentration ranges.

Copper sulfate ($CuSO_4$ – CAS no: 7758-98-7) was used as reference substance to test – monthly – the sensitivity of the organisms, so that it would be possible to certify the health conditions of the tested organisms and hence the toxicity tests validity. The water quality parameters: temperature, water hardness, conductivity and pH were measured at the beginning and at the end of toxicity essays

2.3. Chronic toxicity tests

In the 12 days chronic toxicity tests conducted with the metals copper, manganese, cadmium and mercury and the commercial formula of the pesticides carbofuran and diuron, ten replicates for each treatment were established, one individual in each replicate, and six concentrations of each compound, besides a control, were tested. Both, metals and pesticides tests were performed in plastic cups with one juvenile (average size: 3.75 mm) so it was possible to record its growth, in 10mL of the test solution or 10mL of the reconstituted water (control). Organisms were fed twice during the experiment with 0.5 mL Tetramim® fish feed solution.

For the determination of the concentration gradient, the LC_{20} obtained in the acute tests for each of the referred substances was used, with a division factor of two for the calculation of the lowest concentrations. The following nominal concentrations were used: 0.000625; 0.00125; 0.0025; 0.005; 0.01 and 0.02 mgL^{-1} of copper sulfate;

0.8625; 1.725; 3.45; 6.9; 13.8 and 27.6 mgL^{-1} of manganese sulfate, 0,0141; 0,0281; 0,05625; 0,1125; 0,225 e 0,45 mgL^{-1} of cadmium chloride, 0.003125; 0.00625; 0.0125; 0.025; 0.05 and 0.1 mgL^{-1} of mercury chloride, 0.02346; 0.046875; 0.09375; 0.1875; 0.375 and 0.75 mgL^{-1} of carbofuran commercial formulation and 0.375; 0.75; 1.5; 3; 6 and 12 mgL^{-1} of diuron commercial formulation.

Experiments with both, metals and pesticides, were maintained in darkness at the temperature 22 ± 2.5 °C. Readings were performed on the initial day, on day 3, 5, 8, 10 and 12 (last day). Experimental individuals were observed under a stereomicroscope and the number of dead organisms was counted and the length of each organism (in mm) was recorded.

2.4. Data analysis

For the chronic toxicity tests conducted with the metals copper, manganese, cadmium, mercury and the pesticides carbofuran and diuron both for the mortality and size parameter, the normality (Shapiro-Wilk) and homogeneity of variance of the data (Levene) were verified and differences between treatments were assessed by analysis of variance (ANOVA). This was followed by the post-hoc Fisher LSD test in case of data that met the normality and homoscedasticity criteria. For data that did not meet these requirements, the nonparametric Kruskall-Wallis test, followed by Student-Newman-Keuls post-hoc test, were used. Significant differences between groups were accepted at $p < 0.05$. The analyses were performed using the free Statistica version 7 software (Statsoft, 2004).

3. Results and Discussion

All water bodies show some degree of pollution or chemical imbalance by metals and many of these metals influence the organisms physiological activities without going through the same transformation process of organic compounds, which makes their cycle very slow and makes them susceptible to tissues accumulation in aquatic species (Golovanova, 2008).

Damage to species' survival rates before sexual maturity as well as sub-lethal effects can drastically reduce a population's reproductive success, and consequently its adaptive success. Pollutants can cause qualitative or quantitative decay of gametes, success rate of fertilization, embryonic development, larval viability and, ultimately, species suitability and survival (Au et al., 2001), as observed in the present study. In tropical countries the metal problems and their chronic effects become even greater due to the fact that increasing temperature leads to increased absorption and toxicity of metals, by accelerating metabolic rates, impairing the functioning of mitochondria, leading to stress oxidative damage, damage to the lysosomal system and DNA (Nardi et al., 2017). Aquatic ecosystems integrity is much more threatened by metals and pesticides than terrestrial systems, since aquatic organisms are direct exposure to pollutants and, in these ecosystems, aquatic invertebrates are generally more susceptible to the effects of metal contamination as it has a much higher bioaccumulation potential than vertebrates (Burger, 2008).

For manganese sulfate data on survival and mean length did not show any significant difference (Figure 1 and 2), an explicable result due to the low toxicity of manganese, and the high efficiency that the annelids have in the regulation of metals. Dolagaratz (2018) reported that a sub-lethal effect presented in metal-exposed annelids was the increased secretion of mucus, an adaptive response possibly related to the mucus capacity to complex the metals and decrease their bioavailability, thus composing a mechanism of detoxification. In excess, manganese is cytotoxic and demonstrates increasing levels of reactive oxygen species, impair energy metabolism and deplete glutathione (Martin et al., 2008); Kuperman et al. (2004) observed a decrease in the survival rate of *Eisenia fetida* chronically exposed to manganese, and Norwood et al. (2007) reported a decrease in growth rates to *Hyalella Azteca*.

Because it is an essential nutrient, manganese is actively assimilated by plants and animals with a high bioaccumulation rate at the lowest levels of the aquatic biota, especially in freshwater where, even free of anthropogenic contaminants, it may reach 10 to 10000 µg/L (Martin et al., 2008). Despite the high bioaccumulation factor, manganese is considered one of the least toxic metals, as evidenced in the present study due to the absence of significant variations in the size and survival of both Oligochaeta species.

The mechanisms of copper, mercury and cadmium toxicity act non-specifically on enzymatic sites, and generally involve the blockade of a series of biochemical reactions due to the binding of the contaminating ions to the functional protein groups or by the expulsion of elements of active enzyme centers. Such processes result in damage to cellular metabolism, elevation of lipid peroxidation levels, severe damage to Ca^{2+} homeostasis as well as cell membrane permeability and structure (Golovanova, 2008).

Data obtained for copper sulfate indicated a significant decrease in relation to survival for the last treatment (Figure 1) and in relation to length of the organisms, for the last two (Figure 2). Roman et al. (2007) reported that, for chronic tests of 28 days the presence of copper in low concentrations decreased the growth rate, reproductive capacity and survival rates of *Tubifex tubifex* and *Lumbriculus variegatus*, two species of Oligochaeta recurrently used in ecotoxicological trials, as well as for the species *Hyallela azteca, Chironomus riparius* and *Gammarus pulex*. A decrease in growth rates indicates that less energy is being allocated in production, which may mean that the assimilated energy is being reduced or that this energy is being spent in other physiological processes, such as the elimination of metal, to the detriment of production (Wicklum and Davies, 1996).

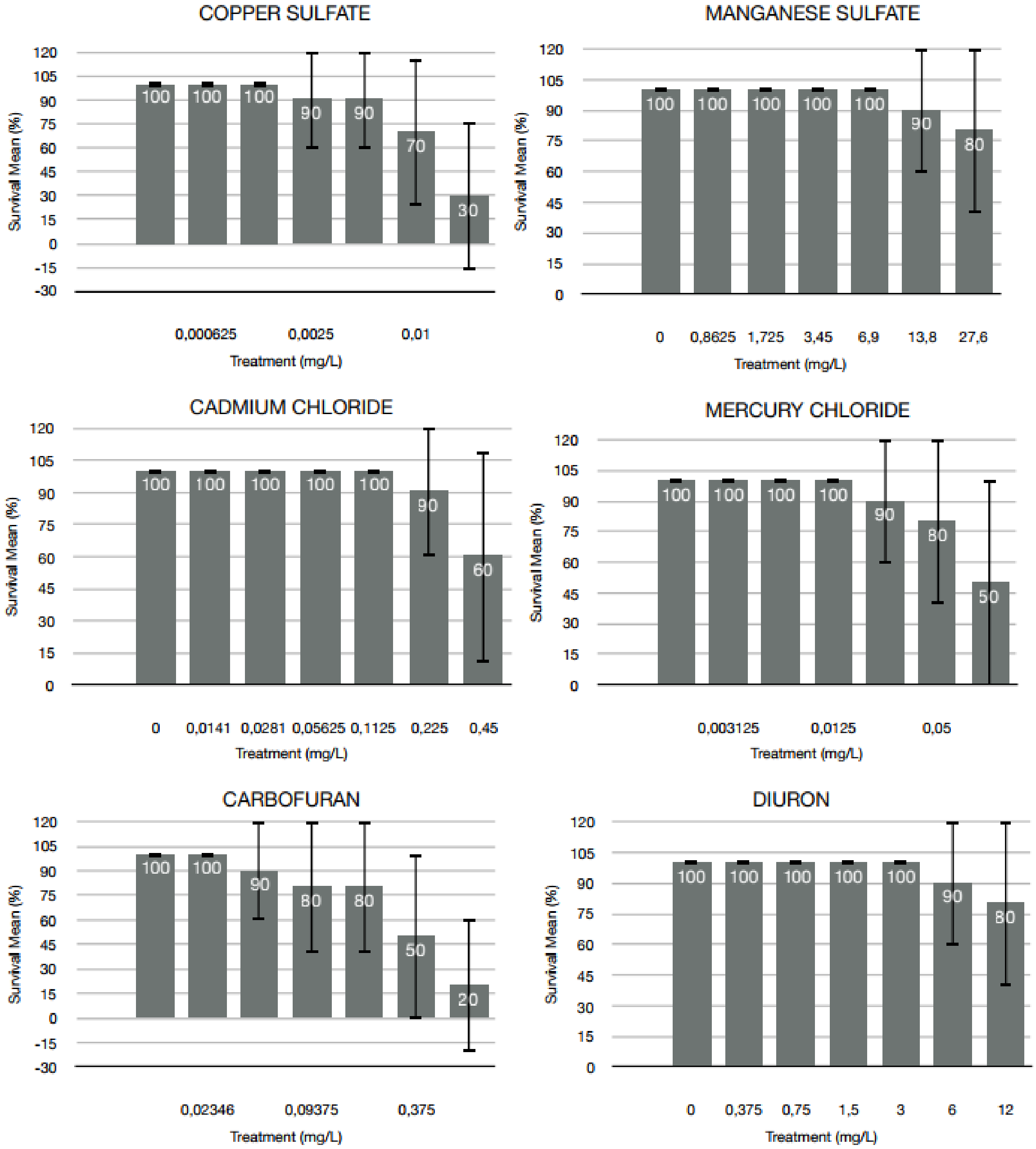

Figure 1. Average values and respective standard deviations for survival at the end of the chronic toxicity test with the *Allonais inaequalis* (Oligochaeta) for the metal compounds copper sulfate, manganese sulfate, cadmium chloride and mercury chloride and to the pesticides carbofuran and diuron (commercial formulations).

For cadmium chloride and mercury chloride, no significant differences in survival data were observed (Figure 1), although the effect of the metal compounds was evident in the highest three concentrations tested for both metals, since the statistical differences indicated a marked decrease in the length of organisms exposed to these contaminants (Figure 2).

Chronic studies with invertebrates exposed to mercury are not recent, in 1981 Gentile reported a decrease in survival rate of 35 and 18% for females and males of the mysid epidemic *Mysidopsis bahia*, respectively, while Lussier et al. (1985) observed a drop in survival rates and of reproduction of the same species.

Marziali et al. (2017) tested the chronic effects of mercury with *Chironomus riparius* and, as in the present study, reported that differences in mortality rates were not significant, although sub-lethal effects were observed in relation to the development rate and lower number of eggs occurred in contaminated sediments. Although dissolved mercury is more bioavailable for aquatic animal consumption, mercury associated with particulate matter tends to sediment in large quantities (Lacerda et al., 2008), which makes it extremely harmful to benthic animals, like *A. inaequalis*, that are in direct contact with this material and consuming part of the sediment.

Although cadmium is a relatively rare metal in the earth's crust and only 4-6% of the cadmium is transferred to aquatic ecosystems, it has a much higher accumulation power in the sediment than in the aquatic biota itself, which gives it a very high toxicity potential (Burger, 2008). In addition to the immediate problem of exposure to cadmium and consequent toxic effects, this metal is not an essential element without known biological function, and it is, therefore, hardly excreted once it is ingested (de Souza Lima Junior et al., 2002), which makes it even more toxic due to the process of bioaccumulation and that causes large damages even in scarce quantities (Holdway et al., 2001). Chronic exposure to cadmium results in metal accumulation in tissues with severe pathophysiological consequences and toxicological effects, and even for vertebrates such as fish, sub-lethal exposures can lead to impairment in growth, survival rates, disruption of ion (Na^+ and Cl^-), oxidative stress and decreased metabolism (Driessnack et al., 2016). Although respiration also participates in the contamination process, the main pathway of cadmium assimilation for aquatic and terrestrial ecosystems is by food, which makes detritivorous and predators invertebrates especially susceptible to cadmium contamination (Burger, 2008; Timmermans et al., 1992)

For other benthic organisms, such as chironomids, Timmermans et al. (1992) and Sildanchandra and Crane (2000) observed that the presence of cadmium may lead to a decrease in larval size, as well as to an increase in the time for emergence of adult chironomids. The authors still observed a decrease in survival, growth and percentage emergence of *Chironomus riparius*, besides reporting absence of reproductive synchrony between males and females, with serious consequences for the insect population.

In addition to the high potential for bioaccumulation of cadmium, Bouché et al. (2000) observed that the uptake of cadmium is fast and efficient by *Tubifex tubifex*, what creates a great risk for its predators. According to these authors, the uptake of metals in aquatic organisms generally dispenses specific mechanisms, and occurs, generally, by passive transport, as shown by Timmermans et al. (1992), from the largest to the lowest metal concentration. For cadmium, part of the transport occurs in an active way, through calcium ion pump. This active mechanism is considered to be responsible for the bioaccumulation, as it goes into action when the cadmium equilibrium is reached. Even with an efficient detoxification mechanism, which allows *T. tubifex* to store large amounts of cadmium, this process can saturate the oligochaetes tissue, causing the so-called spillover, allowing the metal to bind to other proteins and cells, thus inducing toxicity (Klaas R. Timmermans et al., 1992).

Dolagaratz et al. (2018) reported that among the invertebrates, the annelids have a high capacity to accumulate metals, and for *Laeonereis acuta*, a polychaete tested by the authors, it was possible to observe the cadmium accumulation in their tissues even at relatively low concentrations (10 µgCd L^{-1}). The author further states that the accumulation of cadmium appears to occur in both dissolved or particulate forms for sediment and water, which is only possible by the ability to self-regulate by forming insoluble granules of the metal or through metallothioneins binding.

In relation to the pesticides tested, the highest degree of toxicity of the pesticide carbofuran compared to the herbicide diuron is evident, since for the first there were statistical differences, both, in relation to the percentage of survival and the length of organisms, in the last, and in the last two concentrations, respectively (Figures 1 and 2). These effects are similar to those reported by Mansano et al. (2016) in 8-day chronic tests with *Ceriodaphnia silvestrii*, which indicated that both carbofuran commercial formulation and its active principle significantly decreased the cladoceran survival rate on the highest concentration. There were no significant differences between treatments for diuron (Figures 1 and 2), what shows its low level of toxicity, especially in low concentrations. The reduced toxicity of diuron compared to carbofuran to the studied annelid is thus observed; this difference possibly relates to the class of agrochemicals, since carbofuran is a systemic insecticide and nematicide, while diuron is a selective herbicide, which would theoretically bring less severe damage to animals, due to its specific mode of action.

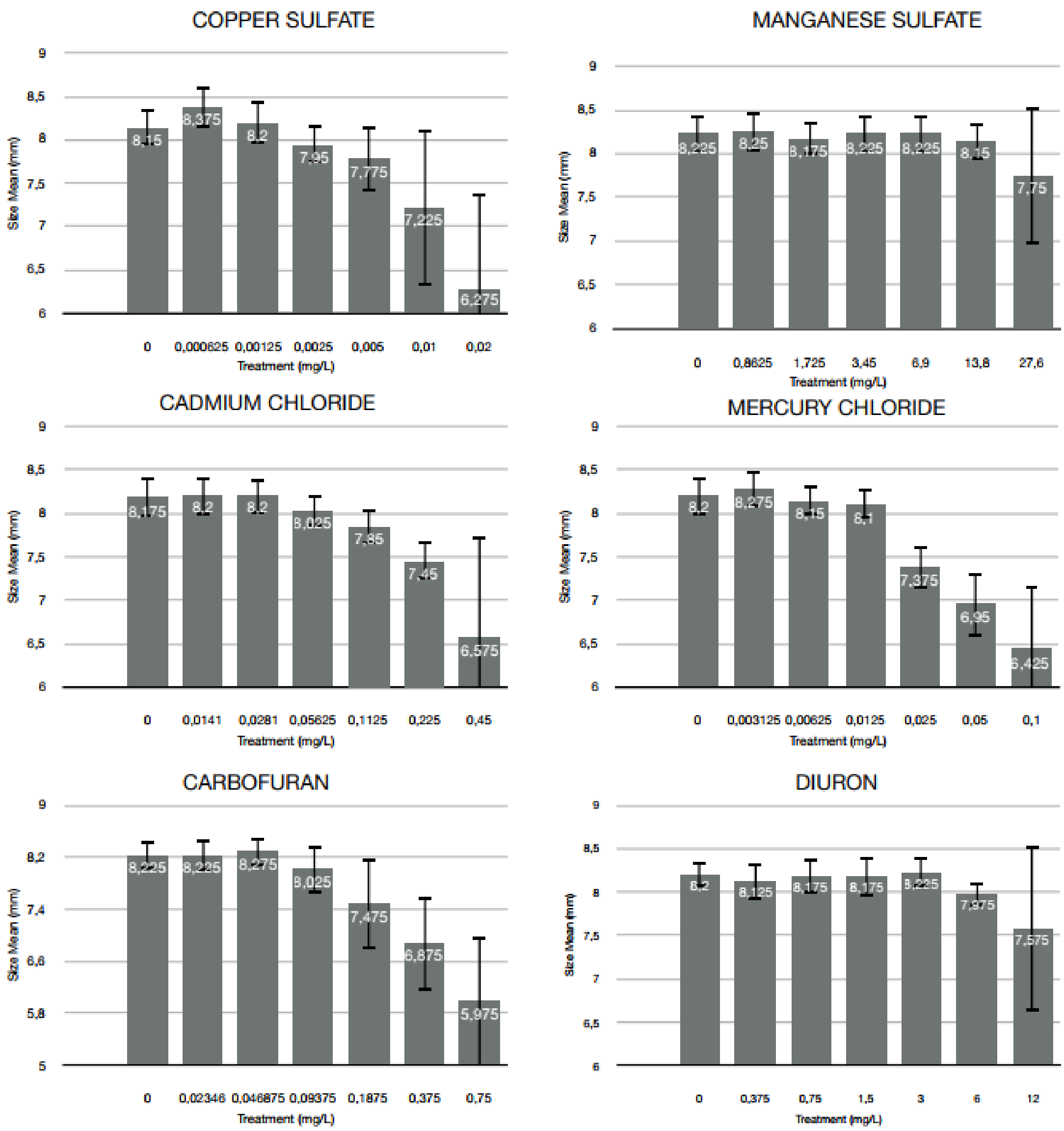

Figure 2. Average values and respective standard deviations for size at the end of the chronic toxicity test with the *Allonais inaequalis* (Oligochaeta) for the metal compounds copper sulfate, manganese sulfate, cadmium chloride and mercury chloride and to the pesticides carbofuran and diuron (commercial formulations).

Thus, it is understood that some elements such as cadmium, copper and mercury present greater ecotoxicological hazard than others such as manganese. At the same time as it can be harmful to the organism, copper and manganese are essential micronutrients as enzymatic cofactors, besides serving as regulators of biochemical functions; biological functions of cadmium and mercury are still unknown (Golovanova, 2008). In both cases, excessive concentrations and constant exposures to these metals may be detrimental to the organism, interfering with survival rates and other endpoints of the species, such as individuals growth reported in the present study, results related to the great potential for bioaccumulation and sensibility of aquatic organisms, especially benthic species.

It can be observed also that residual amounts of pesticides like carbofuran presents lethal effects on invertebrate populations and may present sub-lethal effects that can lead to physiological modifications in organisms that make it impossible to reproduce and maintain the gene pool, besides the possibility to bioaccumulate both compounds (carbofuran and diuron) in the trophic chain and cause problems for the whole community directly or indirectly related to the affected species.

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6. Conclusões e considerações gerais

O presente estudo ressaltou a importância e a viabilidade da utilização de espécies nativas em testes ecotoxicológicos. As espécies testadas, os oligoquetos *Allonais inaequalis* e *Dero furcatus*, tiveram sensibilidade similar ou superior à de espécies de oligoquetos que são internacionalmente utilizadas em protocolos padronizados para ensaios de toxicidade, como *Tubifex tubifex*, *Limnodrilus hoffmeisteri* e *Lumbriculus variegatus,* assim como para espécies de quironomídeos (insetos, dípteros) e dafinídeos (microcrustáceos, cladóceros). Desse modo, pode-se concluir por meio da construção e análise das curvas de distribuição da sensibilidade das espécies, que ambos os anelídeos utilizados tiveram desempenho adequado, sendo bastante sensíveis, podendo ser recomendados como organismos-teste para a realização de ensaios ecotoxicológicos.

Todos os quatro metais testados no presente trabalho apresentaram toxicidade aos organismos analisados em concentrações ambientalmente relevantes: o sulfato de cobre causou maior toxicidade dentre os compostos, seguido pelo cloreto de mercúrio, cloreto de cádmio e sulfato de manganês. A avaliação da toxicidade das misturas de metais mostrou que o sulfato de manganês, embora um composto com toxicidade reduzida, potencializa os efeitos do sulfato de cobre, com evidente efeito sinérgico, o que os torna igualmente danosos à biota aquática. O mesmo efeito sinérgico foi observado para o cloreto de cádmio e o cloreto de mercúrio, o que demonstra que além das consequências diretas da exposição dos organismos aos compostos isolados, é preciso levar em consideração o efeito dos contaminantes quando ocorrendo em misturas, fato recorrente nos sistemas naturais. Desse modo, os ensaios ecotoxicológicos, assim como as avaliações de risco que levem em conta apenas a toxicidade de substâncias isoladas podem não proteger adequadamente a biota e o ecossistema aquático como um todo por subestimar a toxicidade real de poluentes e particularmente de suas interações.

Para os estudos de toxicidade crônica foram demonstrados os severos efeitos sub-letais causados pelo cobre, cádmio e mercúrio sobre a sobrevivência e crescimento dos organismos por um período mais longo, atestando o papel deletério do tempo de exposição ao contaminante no organismo, que provavelmente poderia incluir a bioacumulação. A baixa toxicidade do manganês quando comparado aos outros metais, não o torna menos danoso devido ao seu potencial sinérgico em misturas com outros compostos metálicos, mesmo em exposição por curto prazo, como ficou demonstrado pelos testes de toxicidade aguda com misturas dos metais. Organismos bentônicos como os oligoquetos *D. furcatus* e *A. inaequalis* são especialmente afetados pela toxicidade crônica uma vez que vivem em contato direto com os contaminantes que se acumulam no sedimento dos corpos d´água.

Em relação aos testes de toxicidade crônica com as formulações comerciais dos pesticidas carbofurano e diuron, os resultados aqui obtidos tornaram evidente a elevada toxicidade do primeiro comparado ao segundo, tanto em relação à sobrevivência quanto em relação ao tamanho final dos organismos (crescimento). Este resultado já poderia ser inicialmente esperado, uma vez que o primeiro, carbofurano, trata-se de um inseticida, nematicida e acaricida sistêmico de amplo espectro, com efeitos diretos sobre espécies animais, enquanto o segundo, o diuron, é um herbicida. No entanto, apesar do diuron ter causado baixa mortalidade e alterações no crescimento dos Oligochaeta estudados, ficaram evidenciados seus efeitos sobre os organismos-teste não-alvo, indicando que não se deve descartar seus efeitos sobre populações e sobre a cadeia trófica nos ecossistemas aquáticos de água doce.

Os contaminantes aqui testados apresentaram efeitos letais e sub-letais evidentes, que podem potencialmente afetar não só as populações diretamente em contato com os mesmos, mas toda a biota e ecossistema. Recomenda-se, portanto, que mais testes com estas e diversas outras espécies nativas de oligoquetos sejam realizados com estes e com outros tipos de metais e pesticidas visando produzir um corpo de informações que possam ser amplamente aplicadas na difícil tarefa de proteção à biota e preservação da estrutura e funcionamento dos ecossistemas aquáticos.

Apêndice 1- Resultados obtidos para os testes de sensibilidade aos metais

Tabela 1. Valores médios de LC_{50} 96h e respectivos desvios-padrão para o composto metálico sulfato de cobre resultantes de 20 testes de toxicidade aguda aos organismos-teste *Dero furcatus* e *Allonais inaequalis* (Oligochaeta)

Sulfato de Cobre (mg L^{-1})	*Dero furcatus*	*Allonais inaequalis*
Teste 1	0,02909	0,0249396
Teste 2	0,02957	0,0250918
Teste 3	0,02986	0,0265365
Teste 4	0,03185	0,0257942
Teste 5	0,02900	0,0272967
Teste 6	0,03757	0,0242888
Teste 7	0,03634	0,0250918
Teste 8	0,02824	0,0265167
Teste 9	0,03259	0,0244211
Teste 10	0,02909	0,0279946
Teste 11	0,02957	0,0249396
Teste 12	0,03108	0,0250918
Teste 13	0,02999	0,0242888
Teste 14	0,02986	0,0279946
Teste 15	0,02909	0,0257942
Teste 16	0,03702	0,0256228
Teste 17	0,02986	0,0257942
Teste 18	0,03268	0,0244211
Teste 19	0,02734	0,0272967
Teste 20	0,03802	0,0257942
Média	**0,031384775**	**0,02575049**
Desvio Padrão	**0,003205008**	**0,001151233**

Tabela 2. Valores médios de LC_{50} 96h e respectivos desvios-padrão para os compostos metálicos cloreto de cádmio, cloreto de mercúrio e sulfato de manganês, resultantes de 5 (cinco) testes de toxicidade aguda com os organismos-teste *Dero furcatus* e *Allonais inaequalis* (Oligochaeta)

	Dero furcatus	*Allonais inaequalis*
Cloreto de Cádmio (mg L^{-1})		
Teste 1	0,362388	0,631427
Teste 2	0,38443	0,640025
Teste 3	0,35115	0,597572
Teste 4	0,362388	0,61598
Teste 5	0,360435	0,648414
Média	**0,3641582**	**0,6266836**
Desvio Padrão	**0,010957085**	**0,018081789**
Cloreto de Mercúrio (mg L^{-1})		
Teste 1	0,0895843	0,131567
Teste 2	0,0895615	0,128561
Teste 3	0,0885009	0,132138
Teste 4	0,0942069	0,125614
Teste 5	0,096056	0,128066
Média	**0,09158192**	**0,1291892**
Desvio Padrão	**0,002982388**	**0,002399484**
Sulfato de Manganês (mg L^{-1})		
Teste 1	18,2583	28,1104
Teste 2	18,2712	28,0888
Teste 3	18,3	28,1475
Teste 4	18,2928	28,1581
Teste 5	18,1308	28,271
Média	**18,25062**	**28,15516**
Desvio Padrão	**0,061736356**	**0,063076671**

Apêndice 2- Resultados obtidos para os testes de sensibilidade das espécies aos pesticidas

Tabela 3. Valores médios de LC_{50} 96h e respectivos desvios-padrão para os pesticidas carbofuran e diuron (formula comercial e princípio ativo), resultantes de 5 (cinco) testes de toxicidade aguda com os organismos-teste *Dero furcatus* e *Allonais inaequalis* (Oligochaeta)

	Dero Dero furcatus	*Allonais inaequalis*
	em **(mg L^{-1})**	
Carbofuran Comercial		
Teste 1	0,24745	1,27724
Teste 2	0,267833	1,21839
Teste 3	0,23007	1,21261
Teste 4	0,271098	1,27724
Teste 5	0,247452	1,25482
Média	**0,2527806**	**1,24806**
Desvio Padrão	**0,015064412**	**0,027877065**
Carbofuran Princípio Ativo		
Teste 1	0,309374	1,33635
Teste 2	0,331668	1,50264
Teste 3	0,274867	1,32083
Teste 4	0,305592	1,32083
Teste 5	0,348848	1,42719
Média	**0,3140698**	**1,381568**
Desvio Padrão	**0,025099813**	**0,07233626**
Diuron Comercial		
Teste 1	4,59939	15,1712
Teste 2	4,77067	15,2735
Teste 3	4,44961	16,2489
Teste 4	4,7611	15,1712
Teste 5	4,47353	15,7539
Média	**4,6137275**	**15,52374**
Desvio Padrão	**0,152429898**	**0,421905111**

Diuron Princípio Ativo		
Teste 1	12,9929	19,7538
Teste 2	12,055	21,1504
Teste 3	12,1217	20,4732
Teste 4	12,0512	19,6023
Teste 5	11,6786	20,4041
Média	**12,17988**	**20,27676**
Desvio Padrão	**0,435389855**	**0,556135676**

Apêndice 3- Parâmetros Morfométricos das Espécies

Tabela 4. Medidas de comprimento corporal de indivíduos das espécies *Dero furcatus* e *Allonais inaequalis* (Annelida, Oligochaeta**)** utilizados como organismos-teste em de 5 (cinco) testes de toxicidade aguda em que foram expostos aos pesticidas carbofuran e diuron (formula comercial e princípio ativo).

Tamanho em cm		
	Allonais inaequalis	*Dero furcatus*
1	0,74	0,69
2	0,79	0,73
3	0,82	0,72
4	0,76	0,69
5	0,77	0,71
6	0,73	0,7
7	0,74	0,69
8	0,78	0,73
9	0,77	0,72
10	0,83	0,74
11	0,8	0,72
12	0,75	0,68
13	0,74	0,69
14	0,81	0,7
15	0,83	0,71
16	0,85	0,67
17	0,76	0,66
18	0,84	0,69
19	0,82	0,72
20	0,76	0,71
Média	0,7845	0,7035
Desvio Padrão	0,036942523	0,020560885