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

RAQUEL APARECIDA MOREIRA

EFEITOS DOS AGROTÓXICOS KRAFT® 36 EC (ABAMECTINA) E SCORE® 250 EC (DIFENOCONAZOL), ISOLADOS E EM MISTURAS, SOBRE ORGANISMOS ZOOPLANCTÔNICOS

São Carlos - SP
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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

Co-orientador: Profa. Dr. Michiel Adriaan Daam

Co-orientadora Doutorado-Sanduíche: Profa. Dra. Susana Loureiro

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UNIVERSIDADE FEDERAL DE SÃO CARLOS

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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 da candidata Raquel Aparecida Moreira, realizada em 18/12/2017:

Profa. Dra. Odete Rocha
UFSCar

Profa. Dra. Maria da Graça Gama Melão
UFSCar

Profa. Dra. Marisa Narciso Fernandes
UFSCar

Prof. Dr. Evaldo Luiz Gaeta Espíndola
USP

Profa. Dra. Eny Maria Vieira
USP

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“Quando sentires a saudade retroar fecha os teus olhos e verás o meu sorriso e ternamente te direi a sussurrar: o nosso amor a cada instante está mais vivo!" Guimarães Rosa

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"life and earth evolve together"

(Vida e Terra evoluem juntas)

Croizat, 1964

RESUMO

Os efeitos tóxicos dos agrotóxicos Kraft® 36 EC (i.a. abamectina) e Score® 250 EC (i.a. difenoconazol), isolados e em mistura foram avaliados sobre organismos zooplanctônicos. *Daphnia magna* foi utilizada como organismo-teste para análises dos efeitos por meio dos biomarcadores bioquímicos colinesterase (ChE) e catalase (CAT) e também peroxidação lipídica (LPO), parâmetros relacionados à energia (proteínas e lipídios totais), consumo de energia e testes de toxicidade aguda e crônica. Foram simulados e avaliados os efeitos em ecossistemas aquáticos tropicais por meio de testes de toxicidade com diluições da água de escoamento superficial (*runoff)* contaminada pelos agrotóxicos ao cladócero nativo *Macrothrix flabelligera*. Os riscos relacionados com a exposição aos compostos isolados e em combinação foram avaliados através de experimentação em microcosmos. Por meio de revisão da literatura foi analisada a adequação dos rotíferos para avaliações de efeitos de agrotóxicos nos ambientes aquáticos, com especial ênfase em sistemas tropicais. Constatou-se que existe potencial para espécies nativas serem utilizadas como organismos-teste em avaliações da toxicidade e de risco de agrotóxicos. Uma evidência desse potencial foi a maior sensibilidade destas aos fungicidas quando comparadas ao cladócero *D. magna*. Para *D. magna* os compostos agindo de forma isolada não causaram efeitos sobre a colinesterase (ChE) e catalase (CAT), mas as misturas causaram indução desses biomarcadores. A peroxidação lipídica (LPO) ocorreu quando *D. magna* foi exposta ao fungicida. A reprodução foi pouco e a concentração de proteínas não foi afetada, porém a mais elevada combinação testada para estes agrotóxicos provocou aumento nas reservas lipídicas. A energia consumida foi altamente afetada e estimulada em todos os tratamentos, tanto isoladamente quanto com suas misturas. Para os testes com *M. flabelligera*, as misturas revelaram um desvio dependente da concentração (DL) pelo modelo IA, com antagonismo nas baixas e sinergismo nas altas concentrações da mistura. Os microcosmos que receberam água de *runoff* de solo experimental contaminado dos agrotóxicos individuais não causaram toxicidade para o organismo-teste, porém a mistura causou imobilidade. Os microcosmos tratados por pulverização direta de ambos agrotóxicos resultaram em efeitos tóxicos mais pronunciados. Os resultados sugerem um risco potencial desses compostos em concentrações ambientalmente relevantes, especialmente quando ambos estão presentes.

Palavras-chave: *Daphnia magna, Macrothrix flabelligera*, rotíferos, mistura de agrotóxicos, biomarcadores bioquímicos, reservas energéticas.

ABSTRACT

Toxic effects of the pesticides Kraft® 36 EC (i.a. abamectina) and Score® 250 EC (i.a. difenoconazol), isolated and in mixtures were evaluated on zoplanktonic organisms. *Daphnia magna* was used as test-organism to evaluate pesticide effects using the biochemical biomarkers cholinesterase (ChE), catalase (CAT) and lipid peroxidation (LPO), besides energy-related parameters (total proteins and lipids) together and energy consumption (EC). Possible effects of agrochemicals on tropical aquatic ecosystems were simulated and evaluated by acute and chronic toxicity tests with surface runoff from soil experimentally contaminated with both agrochemicals. The native cladoceran *Macrothrix flabelligera* was used as test-organism. Potential risks related to exposure to isolated compounds and their combinations were evaluated through microcosm experiments. A review of literature was performed to analyze the use of rotifers species as test-organisms for pesticide toxicity evaluation in aquatic ecosystems with emphasis on tropical configurations. It was found that there is great potential for using rotifers as test-organisms for toxicity evaluations and risk assessment. An evidence of this potential was a higher sensitivity of native species exposed to fungicides if compared to *D. magna*. The isolated compounds did not have effects on cholinesterase (ChE) and catalase (CAT), but their mixture induced an increase on these biochemical. Lipid peroxidation (LPO) was induced for *D. magna* was exposed to the fungicide. Reproductive output was small but protein was not quantitatively affected, but the highest combination of both pesticides resulted increased lipid reserves. Consumed energy was highly affected and stimulated in all treatments for both, isolated compounds and mixtures. For *M. flabelligera*, tests with mixtures revealed a concentration dependent deviation (DL) by IA model, with antagonism in low and synergism in high mixture concentrations. The microcosms receiving runoff from experimental soil contaminated with individual pesticides did not cause toxicity to the test-organism, however their mixture caused immobility. The microcosm treated by spray drift with both pesticides showed the highest toxicity effects. Our results suggest potential risk of the selected pesticides in environmentally relevant concentrations, especially if occurring together.

Key-words: *Daphnia magna*, *Macrothrix flabelligera*, rotifers, pesticides mixture, biochemical biomarkers, energy reserves.

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Sumário

Estruturação da tese

Esta tese foi elaborada e estruturada na forma de capítulos, os quais foram redigidos contemplando os itens Resumo, Introdução, Materiais e Métodos, Resultados, Discussão, Conclusões e Referências Bibliográficas de acordo com as normas de formatação de periódicos da editora Elsevier. Assim, além de um capítulo inicial no qual se contextualizou a pesquisa, discorrendo de forma ampla sobre os agrotóxicos (definição, processos, interações e efeitos tóxicos), sobre misturas tóxicas, biomarcadores bioquímicos e estudos ecotoxicológicos por meio de modelos ecossistêmicos (micro/mesocosmos), foram elaborados três outros capítulos, os quais estão ordenados em função do período de desenvolvimento das diferentes etapas da pesquisa, conforme descrito abaixo:

Capítulo 1 - The use of rotifers as test species in the aquatic effect assessment of pesticides in the tropics: Neste capítulo avaliou-se a adequação dos rotíferos como espécies de invertebrados passíveis de serem utilizados como organismos-teste para as avaliações de efeitos de agrotóxicos aos ecossistemas aquáticos, com especial ênfase para ecossistemas tropicais. Embora sejam frequentemente considerados como tendo uma baixa sensibilidade aos agrotóxicos, uma análise de sensibilidade realizada neste estudo revelou que eles podem ser mais sensíveis do que a espécie *Daphnia magna* aos fungicidas. Além disso, poucos dados de toxicidade encontram-se disponíveis para diferentes especies de rotíferos, sendo particularmente restritos a *Brachionus calyciflorus* e esses dados são quase exclusivamente referentes aos testes de toxicidade aguda (CE_{50}). Dada a sua maior diversidade e papel ecológico nas águas tropicais em comparação com as águas doces temperadas, é discutido o uso de rotíferos nas avaliações de risco em região tropical e a necessidade imediata de pesquisa relacionada a esse tema.

Capítulo 2 - Toxicity of abamectin and difenoconazole mixtures to a Neotropical cladoceran after simulated run-off and spray drift exposure: O objetivo deste estudo foi avaliar a toxicidade de Kraft® 36 EC e Score® 250 EC para o cladócero Neotropical, *Macrothrix flabelligera*, uma espécie nativa e de ocorrência comum em águas doces brasileiras. Testes de toxicidade laboratorial foram conduzidos com os compostos individuais para estabelecer seus respectivos limiares de toxicidade. As misturas de ambos os compostos também foram testadas para avaliar o efeito combinado dos produtos. Os riscos potenciais relacionados com a

exposição a ambos os compostos, sozinhos e em combinação, que provavelmente ocorreram no campo através do escoamento superficial e da deriva de pulverização, também foram avaliados através de experimentos em microcosmos.

Capítulo 3 – Effects of abamectin and difenoconazole and their mixtures on *Daphnia magna*: a multiple endpoint approach (biochemical biomarkers, energy reserves and reproduction): Neste estudo, o objetivo foi avaliar a toxicidade do inseticida Kraft® 36 EC (a.i abamectin) e do fungicida Score® 250 EC (a.i difenoconazol) isolados e em misturas sobre *Daphnia magna* em diferentes níveis biológicos. Foram avaliados a sobrevivência (EC_{50}), a reprodução e biomarcadores bioquímicos, colinesterase (ChE) e catalase (CAT) e também a peroxidação lipídica (LPO). Ainda foram estudados os parâmetros relacionados à energia (proteínas e lipídios totais) juntamente com o consumo de energia (EC) via sistema de transporte de elétrons.

Com base nesses três capítulos, as conclusões gerais da tese e as considerações finais foram elaboradas.

1. Introdução e justificativa

1.1. Agrotóxicos: definições e classificações

Uma das formas de maximização da produção agrícola é feita pela adição de compostos químicos às monoculturas para o controle de organismos prejudiciais às culturas, com os chamados agrotóxicos. A partir dos anos 60, o uso destes produtos químicos cresceu exponencialmente devido à chamada Revolução Verde, que, por meio da mecanização e da inserção de formulações de novos produtos, modificou o processo tradicional de produção agrícola (Mazoyer e Roudart, 2010).

De acordo com a Lei Federal 7.802, de 11 de julho de 1989, os agrotóxicos são produtos usados nos setores de produção e beneficiamento de produtos agrícolas, no armazenamento, na proteção de florestas, nas pastagens e de outros ecossistemas. Também são produtos destinados ao uso em ambientes urbanos, industriais e hídricos, com a finalidade de controle da composição da flora ou da fauna, com o intuito de evitar a ação danosa de organismos vivos considerados nocivos, assim como as substâncias e produtos empregados, tais como desfolhantes, dessecantes, inibidores de crescimento e estimuladores (Brasil, 1989). A FAO (*Food and Agriculture Organization of the United Nations*) também tem uma definição própria para estes compostos sendo que a terminologia empregada pelo órgão é *pesticides*, termo comumente utilizado em publicações (OPAS, 1996). Esse termo é definido como qualquer substância ou misturas de substâncias cuja função é destruir, prevenir ou controlar qualquer peste, que estejam causando danos ou interferindo no processo de produção, processamento, estocagem, transporte ou venda de alimentos, *commodities*, madeira e derivados, alimentos para animais, ou substâncias que podem ser administradas em animais para controle de insetos, aracnídeos ou outros parasitas-peste (FAO, 2003).

As primeiras unidades produtivas de agrotóxicos no Brasil datam da década de 1940. No entanto, somente em 1970 foi constituído o parque industrial brasileiro de agrotóxicos (Terra e Pelaez, 2009). Com a implementação da chamada "modernização da agricultura" no Brasil, foi criado o Sistema Nacional de Crédito Rural (SNCR) em 1965 e o Programa Nacional de Defensivos Agrícolas em 1975, importantes iniciativas para a expansão do comércio de agrotóxicos. A partir disso, a indústria e comércio de agrotóxicos cresceu de forma significativa e, desde 2008, o Brasil passou a ser o maior consumidor de agrotóxicos do mundo (Carneiro et al., 2015).

Os agrotóxicos podem ser classificados com base em diferentes critérios, segundo a sua origem, podendo ser inorgânicos (produzidos a partir de compostos de mercúrio, bário, enxofre e cobre), orgânicos (possuem átomos de carbono em sua estrutura) ou de origem vegetal, bacteriana e fúngica (e.g. antibióticos) (Sanches et al., 2003). De acordo com seu modo de ação, os agrotóxicos podem ser classificados em 21 classes, como apresentadas na Tabela 1.

Tabela 1. Classificação de agrotóxicos quanto ao seu modo de ação. Fonte: Modificado de Gavrilescu (2005).

TIPO	MODO DE AÇÃO
Algicidas	Controle de algas
Antimicrobianos	Controle de microorganismos patogênicos
Atrativos	Atraem pragas usando ferormônios naturais que confundem o comportamento de acasalamento de insetos
Avicidas	Controle de aves
Biopesticidas	Substâncias naturais com propriedades pesticidas
Desfolhantes	Causa a queda da folhagem, normalmente para auxiliar no processo de colheita
Dessecantes	Auxilia no processo de secagem de plantas ou insetos
Fumigantes	Produzem vapores ou gases para controle de insetos e doenças comuns ao solo e ar
Fungicidas	Controle de fungos
Herbicidas	Controle de ervas daninhas
Inseticidas	Controle de insetos
Acaricidas	Controle de ácaros
Moluscicidas	Controle de moluscos
Nematicidas	Controle de nematóides e organismos do solo
Ovicidas	Controle de ovos de insetos
Piscicidas	Controle de peixes
Reguladores de Crescimento vegetal	Acelera ou retarda o crescimento vegetal
Praguicidas	Controle de pestes (vertebrados)
Repelentes	Repele mosquitos, carrapatos e outros animais
Rodenticidas	Controle de roedores

Com relação à toxicidade de um agrotóxico, existem quatro classes que devem ser representadas por cores nas embalagens dos produtos. No Brasil, são classificados de acordo

com seu potencial tóxico (Lei nº 7802/1989, regulamentada pelo Decreto nº 4074/2002). Considerando a toxicidade aguda (expressa em DL_{50}), as cores representam as seguintes classificações: vermelho para os produtos considerados extremamente tóxico (Classe I); amarelo para os muito tóxicos (Classe II); azul para os moderadamente tóxicos (Classe III) e verde para os pouco tóxicos (Classe IV) (EMBRAPA, 2003).

Já com relação ao potencial de periculosidade ambiental, os agrotóxicos são classificados em quatro classes: altamente perigoso (Classe I); muito perigoso (Classe II); perigoso (Classe III) e pouco perigoso (Classe IV). A classificação leva em consideração diferentes características dos produtos como, por exemplo, características físicas e químicas, capacidade de persistência e deslocamento no ambiente, bioacumulação nos tecidos vivos e a toxicidade a diferentes organismos (IBAMA, 2009).

Os agrotóxicos também podem ser classificados quanto à sua persistência no ambiente, demonstrando o potencial de contaminação do composto, pois determina o tempo de disponibilidade do produto aos organismos vivos presentes no ambiente. Assim, estes são classificados em: compostos de vida curta (persistência < 5 dias no ambiente); pouco persistentes (de 5 a 15 dias no ambiente); moderadamente persistentes (de 16 a 30 dias) e persistentes (> 30 dias no ambiente) (Hassan, 1997).

1.2. Agrotóxicos: transporte e contaminação

Os agrotóxicos podem chegar ao ambiente aquático por diversas maneiras, envolvendo desde o escoamento superficial em áreas utilizadas para atividades agrícolas e agropecuária, deriva, lixiviação, descarga de efluentes industriais de manufatura de agrotóxicos e de esgoto sanitário, até a aplicação direta para o controle de organismos aquáticos prejudiciais (Zambrone, 1986; Sasal et al., 2010), como demonstrado na Figura 1.

A partir do uso na agricultura, a entrada dos agrotóxicos no ambiente aquático depende, em grande parte, da dinâmica destes compostos no solo. Para compostos com elevada solubilidade em água, o mesmo é facilmente carreado através da lixiviação e escoamento superficial ou infiltração no perfil do solo, vindo a contaminar águas subterrâneas por infiltração. De acordo Schulz (2004), o escoamento superficial é a maior fonte de poluição difusa de agrotóxicos nas águas superficiais. Caso o agrotóxico seja insolúvel em água, o mesmo tende a ficar aderido ao solo e aos materiais orgânicos e inorgânicos que são arrastados

pelo *runoff* (Rieder, 2005). Dessa forma, as características físico-químicas destes produtos, como solubilidade em água, coeficiente de partição n-octanol-água (Kow), pressão de vapor, constante de ionização ácido (pKa), coeficiente de adsorção (Koc) e meia vida, são fatores que determinam o potencial de acumulação do composto em determinado compartimento ambiental ou no organismo de seres vivos, no transporte e, consequentemente, definem o grau de lixiviação, fugacidade e capacidade de retenção (Deuber, 1992).

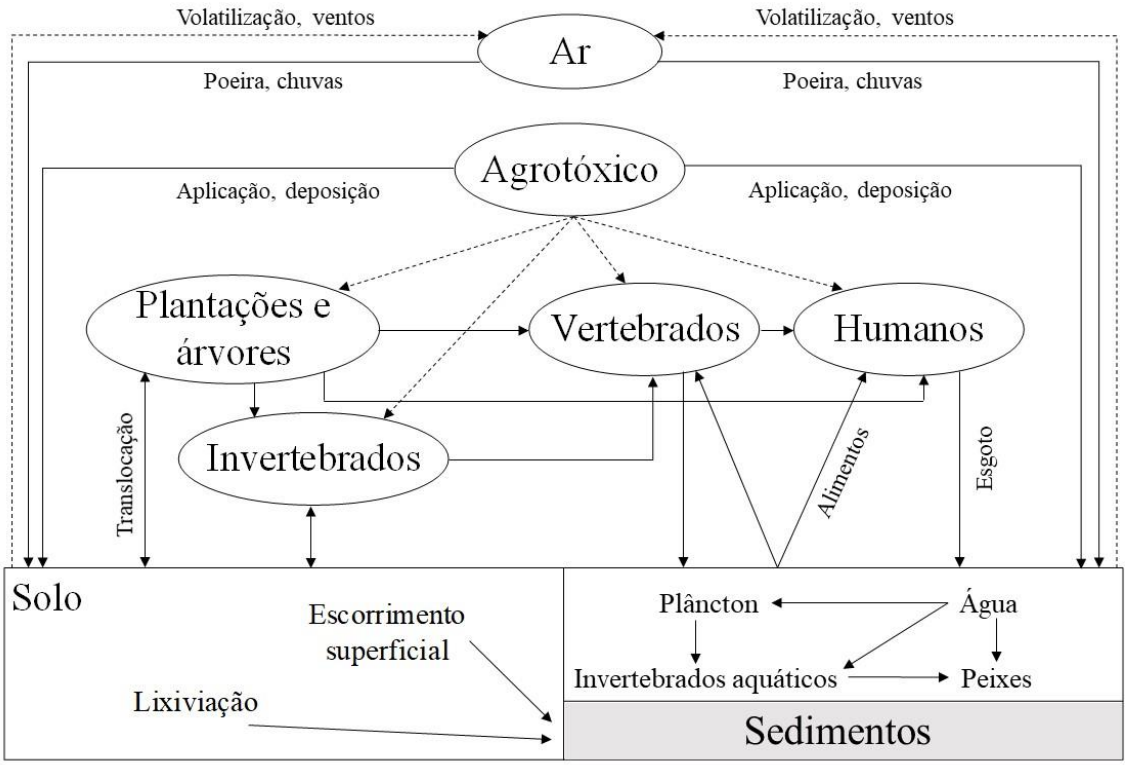

Figura 1. Transporte de agrotóxicos no ambiente. Fonte: Adaptado de Gavrilescu (2005).

No que diz respeito aos efeitos aos organismos, a toxicidade depende das frações disponíveis e acessíveis do produto químico (biodisponibilidade e bioacessibilidade), que variam dependendo da estrutura química, das condições ambientais, da fisiologia dos organismos, das características do ciclo de vida, entre outros fatores (Phyu et al., 2004). A hidrofobicidade/lipofilicidade é uma propriedade físico-química importante que determina a afinidade de uma molécula para ambientes lipofílicos. O conhecimento sobre a lipofilicidade pode indicar se a substância tem potencial para bioacumular, geralmente onde um log Kow > 5 indica um alto potencial de bioacumulação (Coogan et al., 2007). Além disso, os produtos químicos lipofílicos geralmente têm uma meia-vida mais longa. O coeficiente de partição

octanol/água (Kow) é um indicador de lipofilicidade e varia dependendo do pH. Em conjunto com o pH, a temperatura, o teor de matéria orgânica e a dureza são outros fatores físicos e químicos que influenciam o impacto de produtos químicos no meio aquático (Rand e Petrocelli, 1985).

O processo de bioacumulação pode ocorrer pelo contato das espécies com os agrotóxicos, onde os contaminantes são assimilados e retidos nos organismos, causando a ampliação da concentração do contaminante no organismo, aumentando as chances de ocorrência de efeitos deletérios e biomagnificação, que consiste na transferência do contaminante de um nível trófico ao outro, com concentrações crescentes à medida que há a transferência para um nível trófico superior (Buratini e Brandelli, 2006). A atividade microbiológica também pode influenciar nos efeitos tóxicos de contaminantes, aumentando ou diminuindo sua toxicidade e potencial de bioacumulação e interferindo na biomagnificação na cadeia trófica (Zagatto, 2006). Porém, a bioacumulação e biomagnificação dependem de diferentes fatores tais como: a presença de um mecanismo de absorção dos organismos, das propriedades físico-químicas do composto que podem favorecer ou não sua entrada no organismo e da taxa de entrada e depuração do contaminante pelo organismo (Valarini et al., 2003).

A persistência e a mobilidade dos agrotóxicos nos compartimentos ambientais (ar, água, solo, sedimento) depende de variados processos físicos, químicos e biológicos que ocorrem simultaneamente, incluindo retenção (sorção), transporte e transformação (Gavrilescu, 2005; Spadotto et al., 2010). Os agrotóxicos também sofrem modificações durante sua permanência no ambiente. Transformações como adsorção, biodegradação, complexação, fotólise, hidrólise e oxidação-redução são recorrentes e podem transformar os compostos químicos em metabólitos com características diferentes das do produto inicial (Costa et al., 2008). Para alguns produtos, estes metabólitos são ainda mais tóxicos para certos organismos não-alvo que os compostos originais (Beeler et al., 2001).

Condições geoclimáticas também influenciam a dinâmica dos agrotóxicos, como o clima (temperatura, intensidade luminosa, pluviosidade, ventos), a topografia do terreno, assim como condições ambientais, tais como a atividade microbiológica, as propriedades físicas e químicas do solo (teor de matéria orgânica e argila, pH, umidade) e do meio aquático (pH, dureza da água, partículas em suspensão, ácidos húmicos dissolvidos) (Brown et al., 2006; Dores e De-Lamonica-Freire, 1999; Gavrilescu, 2005).

1.3. Agrotóxicos: contaminação aquática e o uso no Brasil

Os ecossistemas aquáticos tropicais constituem grandes reservatórios de biodiversidade, mas se encontram atualmente sujeitos a várias ameaças por serem também receptores temporários ou finais de grande quantidade e variedade de poluentes, sejam eles lançados ao ar, ao solo, ou diretamente nos corpos d'água (Aguilar-Alberola e Mesquita-Joanes, 2012). Quando atingem os ambientes aquáticos, os poluentes podem ter vários destinos, que dependem das propriedades físicas e químicas do composto, das características químicas, físicas e biológicas do ecossistema atingido, bem como da taxa de entrada do composto no ambiente (Rand, 1995), como anteriormente apresentado. Tendo em vista isso, especialmente quando há grande proximidade entre um agroecossistema e ambientes aquáticos, os riscos de contaminação são iminentes (Abhilash e Singh, 2009).

Um exemplo da grande quantidade de produtos utilizados nas monoculturas é o do cultivo de cana-de-açúcar, que em 2011 contava com 62 ingredientes ativos aprovados no Brasil. Destes, 24 são considerados extremamente tóxicos ou altamente tóxicos à saúde humana, 27 são altamente perigosos ou muito perigosos à saúde ambiental, tendo 16 destes apresentado toxicidade aguda aos organismos aquáticos e 38, toxicidade crônica (Schiesari e Corrêa, 2016). Segundo dados do Sistema de agrotóxicos fitossanitários, em 2017 já são 85 ingredientes ativos aprovados, compondo 441 produtos formulados (AGROFIT, 2017). As formulações comerciais podem apresentar toxicidade bastante diferente aos organismos, uma vez que possuem uma série de outros ingredientes (chamados inertes) que não são estudados separadamente por serem desconhecidos. Desta forma, o impacto do aumento da liberação de agrotóxicos no ambiente aquático é notado pela perda da biodiversidade e da qualidade dos recursos hídricos. Por isso, os estudos de monitoramento de resíduos de agrotóxicos têm aumentado ano a ano e têm indicado que resíduos destes produtos estão presentes nos alimentos (ANVISA, 2017), na atmosfera (Moreira et al., 2012), nas precipitações secas e úmidas, como chuvas (Nogueira et al., 2012) e nas águas superficiais e subterrâneas (Dores et al., 2009).

A grande utilização de agrotóxicos no processo de produção agrícola, na agropecuária, dentre outras aplicações, tem trazido uma série de transtornos e modificações para o ambiente, seja pela contaminação das comunidades de seres vivos que o compõem, seja pela acumulação nos segmentos bióticos e abióticos dos ecossistemas (biota, água, ar, solo, sedimentos, etc) (Peres, Moreira e Dubois, 2003). Um dos maiores problemas relacionados com o uso dos

agrotóxicos é que, por não serem completamente seletivos, estes atingem espécies não-alvo, representando, portanto, uma ameaça aos ecossistemas (Schreinemachers e Tipraqsa, 2012).

Os agrotóxicos afetam ainda a dinâmica das populações (reprodução, migração, restabelecimento e mortalidade), a estrutura e função das comunidades (alteração na diversidade de espécies, modificações na relação predador-presa) e alteram os serviços ecossistêmicos (alterações nos processos de respiração e fotossíntese e no fluxo de nutrientes) (Costa et al., 2008; Silva et al., 2013). Os efeitos ocasionados à saúde humana também estão intimamente relacionados à contaminação aquática por agrotóxicos, como, por exemplo, os neurológicos (Meyer-Baron et al., 2015; Wesseling et al., 2002), mutagênicos (Miligi et al., 2006; Saleem et al., 2014) e perda de qualidade da água como um recurso para o ser humano (Silva e Santos, 2007).

Os agrotóxicos podem causar efeitos nocivos a diferentes níveis de organização biológica. Em níveis de organização mais simples (e.g. respostas moleculares e celulares, alterações bioquímicas e fisiológicas), os efeitos acontecem de maneira mais rápida e, por isso, biomarcadores bioquímicos são importantes ferramentas como sinalizadores de exposição a contaminantes. Em níveis superiores de organização (indivíduo, população e comunidade), diversos estudos comprovaram impactos negativos dos agrotóxicos, como a redução do número de espécies, alterações comportamentais, reprodutivas e de desenvolvimento, suscetibilização a doenças, distúrbios em processos ecológicos, como por exemplo, na atividade de decomposição, que consequentemente afetará a sustentabilidade da cultura ou do ambiente em longo prazo (e.g. Choung et al., 2013; Mansano et al., 2016; Novelli et al., 2012; Nunes e Espíndola, 2012).

A maioria dos estudos sobre a toxicidade e avaliação de risco de agrotóxicos para os ecossistemas aquáticos foi realizado em países da região temperada (Lacher e Goldstein, 1997; Racke, 2003). Consequentemente, as avaliações de risco aquático nos países tropicais geralmente dependem de dados de toxicidade de espécies exóticas (Kwok et al., 2007), como os cladóceros frequentemente utilizados em protocolos padronizados, *Daphnia magna* e *Ceriodaphia dubia*, embora o destino e os efeitos dos agrotóxicos possam diferir entre regiões climáticas (Daam e Van den Brink, 2010). Isso implica pouco realismo ecológico das atuais avaliações de risco em região tropical e o risco estimado com base em valores que não ocorrem sob as condições tropicais (Garcia, 2004; Silva e Van Gestel, 2009). Estudos com uso de espécies nativas demonstraram que estas podem ser mais sensíveis do que os organismos-teste de ambientes temperados (por exemplo, Buch et al., 2017; Freitas e Rocha, 2012; Ghose et al.,

2014; Howe et al., 2014; Mansano et al., 2016; Moreira et al., 2014, 2017; Silvano e Begossi, 2016).

As condições geoclimáticas de cada ecossistema influenciam diretamente nos efeitos tóxicos dos agrotóxicos aos organismos não-alvo (Sanchez-Bayo e Hyne, 2011), como apresentado nas seções anteriores. Além disso, outros fatores podem influenciar, como a maior frequência de aplicação e durante uma maior parte do ano em países de regiões tropicais, em comparação com aqueles de regiões temperadas (Ecobichon, 2001; Satapornvanit et al., 2004). Esta exposição repetida pode dificultar a recuperação dos organismos afetados nos intervalos entre as aplicações, levando a comunidades cronicamente alteradas (Daam e Van den Brink, 2010). A aplicação via *spray-drift* pode ser mais severa nos trópicos porque agrotóxicos são frequentemente aplicados muito perto dos ecossistemas aquáticos (Daam e Van den Brink, 2010).

A regulamentação do uso de agrotóxicos e a caracterização de seus riscos geralmente se baseiam em estudos que se concentram principalmente na sensibilidade de uma variedade de espécies representativas de diferentes níveis tróficos para um único produto químico. Em ambientes naturais, as espécies estão constantemente expostas a misturas complexas de agrotóxicos com constituintes adjuvantes variáveis e em diferentes concentrações e proporções, o que pode levar a efeitos aditivos ou produzir efeitos sinérgicos ou antagônicos (Cedergreen et al., 2008; Faust et al., 2003, Mansano et al., 2017). Além disso, embora a maioria dos estudos avalie apenas a toxicidade dos ingredientes ativos de agrotóxicos, as formulações comerciais incluem, além de ingredientes ativos, surfactantes e outros ingredientes inertes, que podem contribuir significativamente para a toxicidade geral destes produtos (Cedergreen e Streibig, 2005; Moreira et al., 2017). Por isso é crucial investigar os efeitos tóxicos dos ingredientes ativos de agrotóxicos em uma variedade de espécies, bem como os efeitos tóxicos relacionados com suas formulações comerciais, em exposições individuais e em mistura, a fim de fornecer informações mais realistas sobre os impactos tóxicos de agrotóxicos em organismos sensíveis não-alvo.

1.4. Os agrotóxicos Kraft® 36 EC (i.a. abamectina) e Score® 250 EC (i.a. difenoconazol)

A composição da formulação comercial Kraft® 36 EC (Cheminova Brasil Ltda, 2017) é 36% m/v de ingrediente ativo (i.a), a abamectina e 69,4% m/v de ingredientes inertes. Quanto a sua toxicidade, este agrotóxico é classificado pela ANVISA (2017) como extremamente

tóxico a saúde humana (Classe 1) e como muito tóxico ao meio ambiente pelo IBAMA (2017) como classe 2 (Figura 2A).

A abamectina pertence ao grupo químico das Avermectinas (Fisher e Mrozik, 1989). As avermectinas são lactonas macrocíclicas, derivadas da fermentação da bactéria do solo *Streptomyces avermitilis*. Nesse grupo encontram-se a ivermectina, a abamectina e a doramectina. A ivermectina foi lançada no mercado como uma droga antiparasitária em 1981, e anos mais tarde, em 1985, a abamectina foi introduzida como inseticida e acaricida na agricultura, e também como antiparasitário em criação de animais. A abamectina é uma mistura que contém cerca de 80% de avermectina B1a e 20% da avermectina B1b, e estes dois componentes têm propriedades biológicas e toxicológicas similares (Campbell, 1989; Lankas e Gordon, 1989; Fisher e Mrozik, 1992). Quanto à sua funcionalidade ela é classificada como um inseticida/acaricida/nematicida de origem biológica, além de ser um agente antiparasitário em animais de criação e estimação (Kolar et al., 2008). O principal modo de ação das avermectinas é sobre o sistema nervoso dos organismos, em artrópodes e nematódeos. A interação com os receptores GABAérgicos presentes na membrana pós-sináptica estimula o fluxo de íons cloreto para o interior das células nervosas causando a interrupção dos sinais neurais (Tišler e Erţen, 2006), sendo considerado altamente tóxico para abelhas e organismos aquáticos.

Este agrotóxico pode ser usado em diversas culturas como algodão, batata, feijão, maçã, mamão, citros, crisântemo, tomate e morango. Nesta última, o produto formulado costuma ser aplicado para erradicação do ácaro-rajado da espécie *Tetranychus urticae*. Segundo a bula, respeitando-se a dose de 25 a 30 mL a cada 100L de água aplicada na plantação e um máximo de duas a três aplicações intervaladas de sete dias.

O agrotóxico Score® 250 EC (Syngenta Proteção de Cultivos Ltda, Brasil, 2017) tem na composição de sua formulação 250% m/v de ingrediente ativo (i.a.), o difenoconazol e 750% m/v de ingredientes inertes. Quanto a sua toxicidade, assim como o Kraft® 36 EC é classificado pela ANVISA (2017) como extremamente tóxico a saúde humana (Classe 1) e como muito tóxico ao meio ambiente pelo IBAMA (2017) como classe 2, além de ser considerado altamente persistente no meio ambiente (Figura 2B).

O difenoconazol pertence ao grupo químico dos triazóis. Nesse grupo, também encontram-se o ciproconazol, epoxiconazol, metconazol, flutriafol, propiconazol, protioconazol, tebuconazol e tetraconazol. Os triazóis são muito específicos em seu modo de ação, inibem a biossíntese do esterol, um componente crítico para a integridade de membranas celulares dos fungos (Fishel, 2005). Introduzido na década de 1980, o difenoconazol é um

fungicida sistêmico, importante contra doenças de gramados, vegetais, frutas cítricas, culturas, plantas ornamentais e diversas olerícolas. É utilizado também no tratamento de sementes contra diversos patógenos de trigo e cevada (Ehr e Kemmitt, 2002). Este composto, inibe a biossíntese de ergosterol, ao impedir a desmetilação dos esteróis. Este modo de ação interfere com as funções da membrana celular e com o crescimento micelial do fungo, mas pouco influencia a germinação dos conídios e o desenvolvimento dos haustórios (Tomlin, 1994). É altamente tóxico para organismos aquáticos, sendo também classificado como poluente marinho severo (Syngenta, 2017).

Este fungicida pode ser usado como agrotóxico em diversas culturas como algodão, alface, abobrinha, alho, amendoim, arroz, banana, batata, berinjela, beterraba, café, cenoura, cebola, citros, couve-flor, ervilha, feijão, maçã, mamão, manga, maracujá, melancia, melão, pepino, pimentão, soja, uva e morango. Nesta última, o produto formulado costuma ser aplicado para erradicação da mancha mycosphaerella causada pelo fungo *Mycosphaerella fragariae*, segundo a bula, respeitando-se a dose de 40 mL a cada 100 L de água aplicada na plantação com intervalos mínimos de 14 dias.

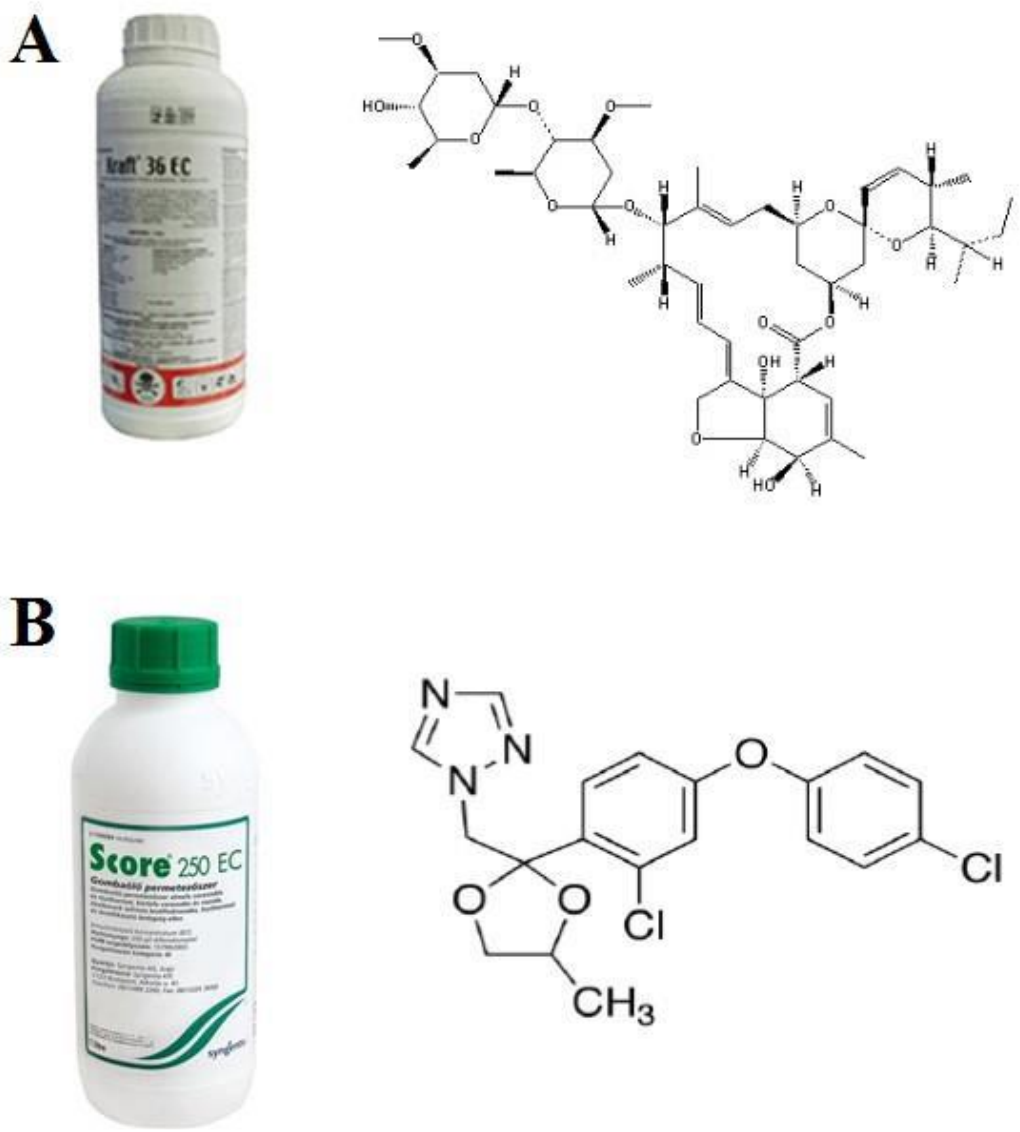

Figura 2. Fórmula estrutural da abamectina (A) e do difenoconazol (B) juntamente com a imagem de seus respectivos produtos comerciais utilizados nos experimentos. Fonte: Cheminova, 2017 e Syngenta, 2017.

1.5. Misturas tóxicas

Alguns aspectos da contaminação do ambiente por agrotóxicos estão relacionados com os diferentes e excessivos modos de aplicação no campo (Shinn et al., 2015). Diversos produtos que são muito tóxicos são oficialmente registrados e licenciados para uso em algumas culturas as quais não têm outro produto para o controle das pragas, porém, devido à sua alta eficácia, são aplicados em outras culturas para as quais não são recomendados. Além disso, buscando ainda maior eficiência, mais de um produto são por vezes aplicados simultaneamente e em doses maiores que as recomendadas pelo fabricante (Dellamatrice e Monteiro, 2014) e por isso, atualmente, estudos com agroquímicos também estão levando em consideração as misturas de

contaminantes que possivelmente ocorrem nos ecossistemas aquáticos (Hoagland et al., 1993; Cuppen et al., 2002; Van Den Brink et al., 2002, 2009; Adam et al., 2009; Zhou et al., 2010). Um exemplo disso é verificado no município de Bom Repouso, Estado de Minas Gerais (Figura 3), onde os agricultores, nas plantações de morangos e batatas, dentre outras, utilizam demasiadamente dois agrotóxicos, em especial o Kraft® 36 EC, cujo princípio ativo é a abamectina, e o Score® 250 EC, que tem como princípio ativo o difenoconazol (Nunes e Espíndola, 2012) (Figura 4).

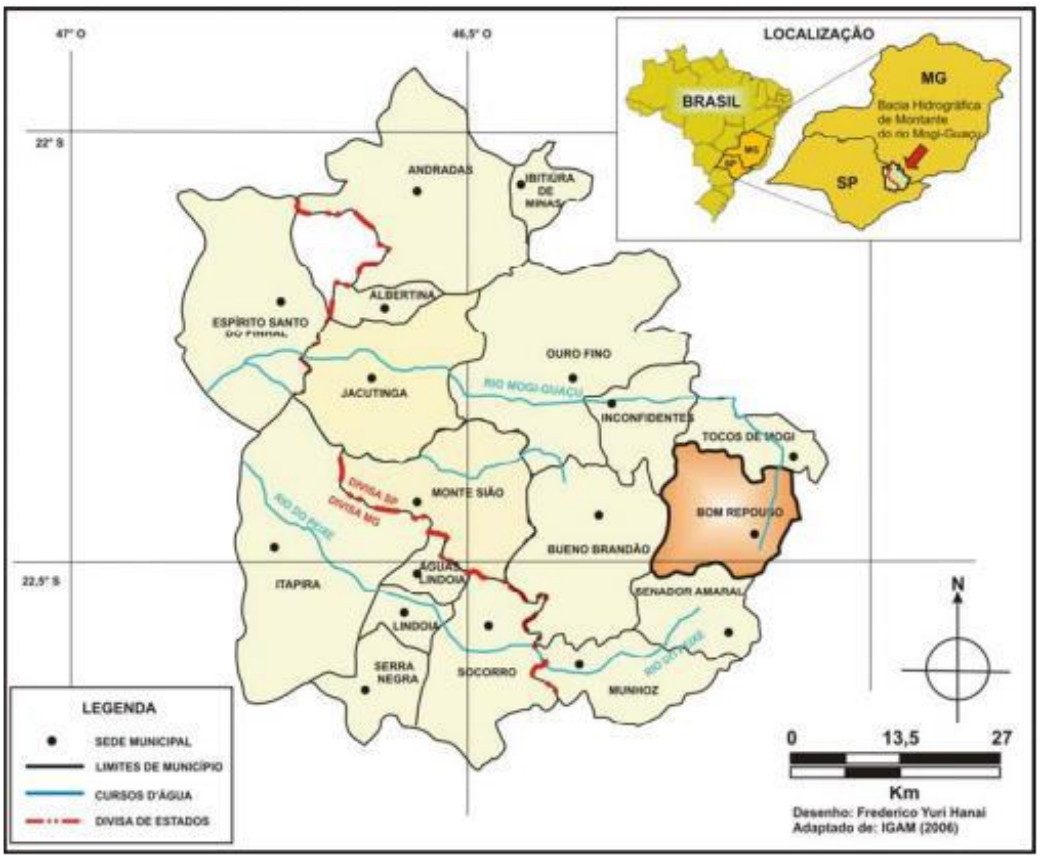

Figura 3. Localização do município de Bom Repouso, Minas Gerais (em destaque) e municípios adjacentes. Fonte: Espíndola e Brigante (2009).

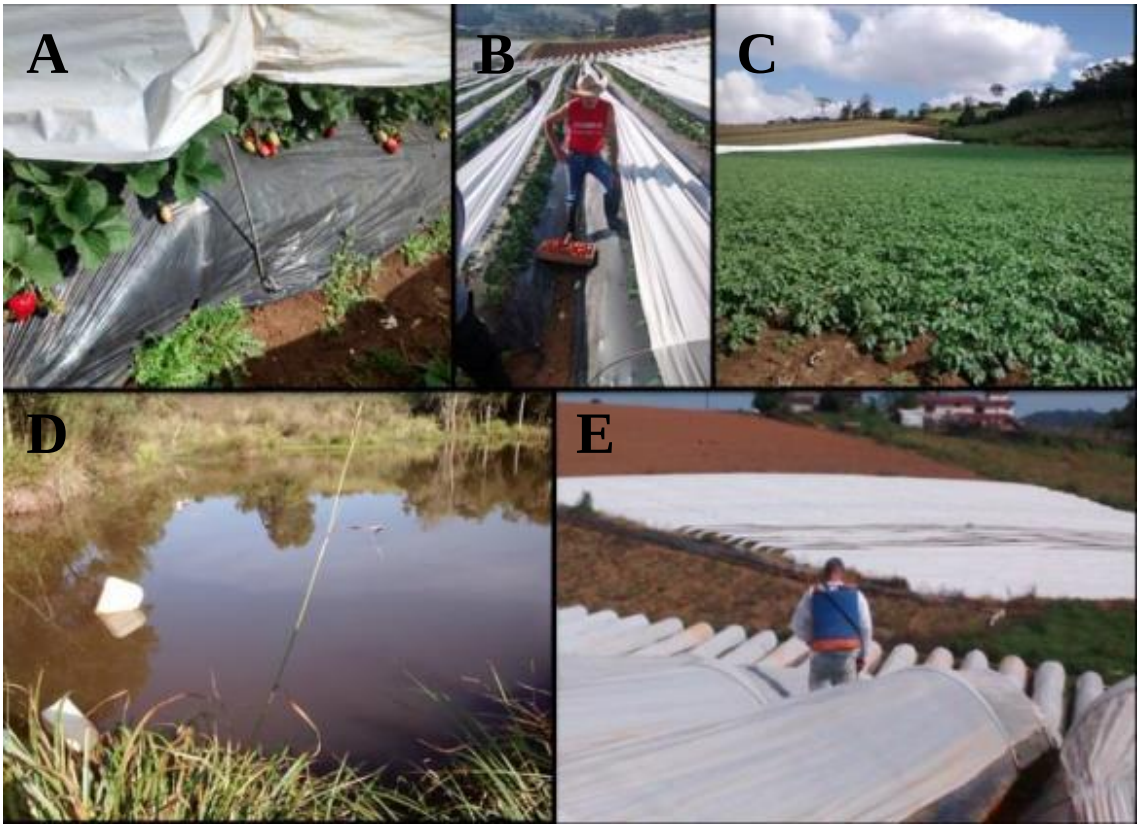

Figura 4. Produção agrícola de Bom Repouso (MG). A), B) plantações de morangos; C) plantação de batata; D) descarte inadequado de embalagens de agrotóxicos e E) manipulação inadequada do agrotóxico. Fotos: Marina Reghini Vanderlei, 2014.

Reconhecendo-se que nos ecossistemas naturais os organismos são frequentemente expostos a um coquetel de produtos químicos, um desafio importante é estimar os efeitos interativos de produtos químicos para melhorar a avaliação de risco ambiental. Além disso, o *Framework for Cumulative Risk Assessment* ressalta a necessidade de analisar os riscos combinados para a saúde humana de múltiplos estressores ao meio ambiente (USEPA, 2003).

O modo de ação (MOA) de um produto químico é definido como um conjunto de sinais bioquímicos, fisiológicos e/ou comportamentais gerados da exposição de um organismo a um estressor (McCarty, 2002) e é um importante item a ser considerado nos testes de misturas tóxicas. O conceito de MoA é diferente em Toxicologia humana e Ecotoxicologia, sendo a última mais ampla e envolvendo diferentes efeitos tóxicos para a comunidade biológica (microorganismos, animais - invertebrados e vertebrados e plantas) (EFSA, 2015). Além disso, um único produto químico pode ter diferentes modos de ação e, com isso, a complexidade na identificação do MoA é um desafio na previsão dos efeitos da mistura (EFSA, 2015), especialmente a mistura de produtos formulados de agrotóxicos, que por si só, abrange a mistura de diferentes produtos, o ingrediente ativo e ingredientes inertes.

Na avaliação de risco atual, a avaliação dos efeitos combinados de produtos químicos geralmente pode ser prevista utilizando-se dois modelos conceituais, os modelos de Ação Independente (IA) (*Independent Action* - IA) (Bliss, 1939) e de Adição de Concentração (CA) (*Concentration Addition* - CA) (Loewe e Muischnek, 1926). Ambos os modelos são baseados na não interação entre químicos/estressores, assumindo que cada componente não interage/influencia a ação biológica do outro componente dentro de uma mistura (Hewlett e Plackett, 1959).

O modelo IA é usado para substâncias com modos de ação diferentes e a formulação matemática é baseada na probabilidade de respostas e é expressa da seguinte forma:

$$Y = \mu \max \Pi_{i=1}^{n} qi\ (Ci)$$

Onde Y denota a resposta biológica, Ci é a concentração de química i na mistura, *qi* (*Ci*) a probabilidade de não resposta, μ max a resposta de controle para parâmetros analisados e Π a função de multiplicação.

Por outro lado, o modelo de CA geralmente é usado para substâncias de atuação similar (MoA similar), onde os componentes assumem que atuam como diluições dos outros e, matemáticamente, podem ser expressos como:

$$\sum_{i=1}^{n} \frac{Ci}{ECxi} = 1$$

Onde ci denota a concentração para o produto químico i na mistura e ECxi é a concentração de efeito do produto químico i que resulta no mesmo efeito que a mistura. Este quociente ci/ Cxi é comumente referido como o valor da Unidade Tóxica (TU), representando a contribuição para a toxicidade individual do químico i em uma mistura.

Para o propósito de avaliação de risco, segundo Cedergreen et al. (2007), o modelo de adição de concentração (CA) é frequentemente preferido por ser, em geral, mais conservativo. Porém, são relatados na literatura exemplos em que os modelos de adição de concentração e/ou de ação independente previram de forma satisfatória a toxicidade da mistura de determinados estressores (e.g. Backhaus et al., 2004; Cleuvers, 2005; Faust et al., 2003; Loureiro et al., 2010).

Uma vez que os produtos químicos podem interagir dentro dos organismos, podem ocorrer desvios de ambos os modelos de referência, como sinergismo ou antagonismo, relação

de dose ou dependência de nível de dose (Jonker et al., 2005; Loureiro et al., 2010). Através da ferramenta computacional MIXTOX, esses desvios podem ser avaliados (Jonker et al., 2005). O primeiro desvio corresponde ao sinergismo ou antagonismo, sendo o efeito sinérgico caracterizado por um efeito maior que o aditivo (efeito mais severo) e o efeito antagonista caracterizado por um efeito menor do que aditivo (efeito menos grave) (Bliss, 1939). No desvio de nível de dose (DL) (*Dose Level* - DL), a toxicidade depende da dose e difere em doses baixas ou altas dos produtos químicos. No desvio dependente da razão de dose (DR) (*Dose Ratio* - DR), a toxicidade depende da composição da mistura e é causada principalmente por um dos produtos químicos (Jonker et al., 2005). Estes desvios em relação aos modelos de referência podem ser de natureza física, química e/ou biológica e as interações podem ocorrer na fase de toxicocinética (processos de absorção, metabolismo, distribuição e excreção) ou na fase de toxicodinâmica (efeitos do químico nos receptores, sítios celulares ou órgãos) (Cassee et al., 1998; Ferreira et al., 2008).

O isobolograma é a representação gráfica comumente utilizada para as interações de compostos nas misturas tóxicas, especificamente, de doses isoladas, e em combinação, que causam uma porcentagem de efeito (e.g. X = 50%, CE_{50}) de dois compostos. Um isobolograma é representado por isoboles de aditividade, sinergismo e antagonismo (Figura 5). No eixo X, são apresentadas as doses do composto A e no eixo Y as doses do composto B, sendo que cada ponto representa um par de doses que atingem o CE_{50} quando estão associados. Como observado na figura, os CE_{50} gerados da aplicação isolada de cada produto são unidos, gerando a isobole de aditividade. Os outros valores de CE_{50} obtidos da associação em diferentes proporções dos produtos podem ser analisados em relação à sua posição diante da isobole de aditividade. No caso de pontos que ficam posicionados em torno da isobole de aditividade, a ação é de aditividade; para sinergismo ficam localizados abaixo e se estiverem localizados acima, a ação é de antagonismo. Assim, não interação, sinergismo e antagonismo são representados por isoboles linear, côncava e convexa, respectivamente (Kruse et al., 2006; Ryall e Tan, 2015).

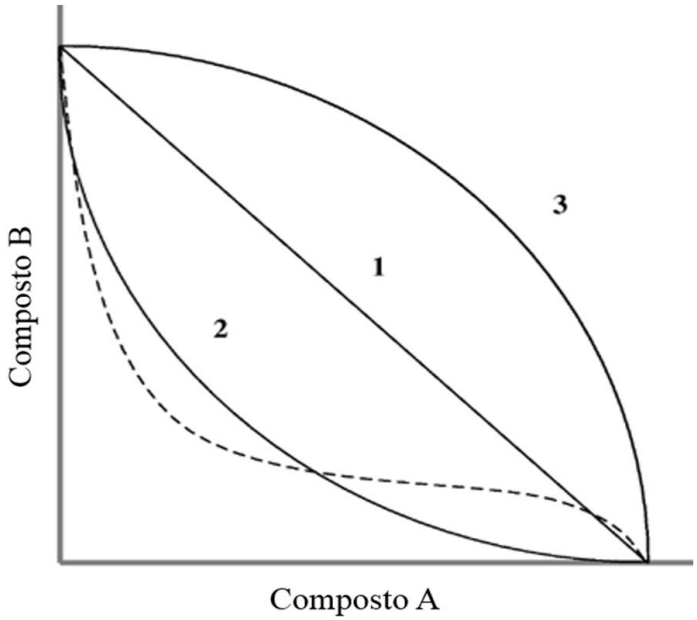

Figura 5. Representações das interações entre os compostos A e B são representadas pelo isobolograma, onde: aditividade (sem interação) é apresentado pela curva 1, sinergismo pela curva 2 e antagonismo pela curva 3. Uma isobole enviesada é representada pela linha pontilhada. Fonte: Modificado de Bell (2005).

Além da exposição constante ou da exposição à mistura, a exposição episódica a produtos químicos ocorre, por exemplo, após a liberação acidental e após a aplicação de agrotóxicos e/ou escoamento superficial. Isso pode resultar em exposições de pulso a curto prazo, com produtos químicos que entram no ambiente em diferentes momentos (Liess et al., 1999). Por esses motivos, os organismos aquáticos que vivem em áreas adjacentes podem sofrer exposições episódicas por diferentes períodos de tempo. Esses pulsos químicos são dinâmicos e podem ocorrer sozinhos ou em mistura e durar horas a dias (Gordon et al., 2012). Além disso, os efeitos tóxicos dos pulsos químicos dependem da concentração do produto químico, geralmente com baixas concentrações que apresentam uma possível recuperação após a exposição ao estressor (Gordon et al., 2012). Diante disso, pulsos químicos devem ser melhor estudados, para melhor compreensão de processos de misturas para avaliação de riscos ambientais.

1.6. Biomarcadores bioquímicos

Os biomarcadores bioquímicos têm sido empregados em diversos estudos como ferramentas funcionais para avaliar a toxicidade de uma variedade de compostos sobre as populações naturais (Rodrigues et al., 2011). Em Ecotoxicologia, os biomarcadores podem ser definidos como marcadores de alterações em uma resposta biológica (desde mudanças moleculares por meio de

respostas celulares e fisiológicas, a mudanças comportamentais) que refletem os efeitos tóxicos de contaminantes dispersos no ambiente (Peakall, 1994; Islas-Flores et al., 2013).

Segundo Depledge et al. (1993), Timbrell et al. (1996) e Walker et al. (2001), os biomarcadores podem ser divididos nas três seguintes classes:

• Biomarcadores de exposição: são alterações biológicas verificáveis que evidenciam a exposição dos organismos a um contaminante, mas não dão informações do grau de efeito adverso que estas mudanças causam nos organismos. Uma das grandes vantagens do uso desses biomarcadores é o custo, que é bastante inferior comparado ao custo de análises químicas (Schlenk, 1999). A indução das metalotioneínas por metais é um exemplo de um biomarcador de exposição.

• Biomarcadores de efeito: Estes biomarcadores são caracterizados pela indução de mecanismos de defesa celular, que se iniciam como uma resposta adaptativa em nível molecular-bioquímico. Porém, se esse mecanismo falha ou se sua capacidade de resposta é extrapolada, poderão ser provocadas alterações fisiológicas ou histológicas, que podem ser irreversíveis, dependendo da capacidade do sistema ou órgão em responder ao estressor, demonstrando assim, efeitos adversos de um tóxico em particular, ou grupo de tóxicos nos organismos. Dessa forma, o organismo pode, por exemplo, ter sua capacidade de reprodução ou crescimento afetada (Winzer et al., 2001). A inibição da acetilcolinesterase pelos agrotóxicos organofosforados e carbamatos é considerado um importante biomarcador bioquimico especifico de efeito.

• Biomarcadores de susceptiblidade: biomarcadores indicadores de processos que causam variações de respostas ao longo do tempo e entre exposição e efeito (Barret et al., 1997), determinando condições como: indivíduo sadio, compensação do metabolismo, perturbação das funções, alterações morfológicas e morte. Os organismos, mesmo sendo da mesma espécie, não respondem da mesma forma à exposição aos contaminantes. Sexo, jejum, estresse pelo confinamento, tamanho e estádio de desenvolvimento são parâmetros de variação nas respostas a estressores. Estes biomarcadores correspondem aos biomarcadores de efeito latente (Fossi e Leonzio, 1993), no qual, um organismo pode, em determinadas circunstâncias, ter limitada a habilidade de se adaptar ou sobreviver, o que pode ser indicado por respostas fisiológicas que, analisadas em conjunto, demonstram a diminuição da energia disponível para o crescimento (Nascimento et al., 2006).

Outra classificação dos biomarcadores bioquímicos (Peakall, 1992) defendida por alguns autores que consideram a classificação anterior limitada, do ponto de vista fisiológico ou funcional, é dividida em:

- biomarcadores de enzimas de biotransformação, indução vs. inibição das atividades das enzimas de biotransfomação após exposição a um xenobiótico, como por exemplo a glutationa S-transferase e as UDP -glicuroniltransferases;
- biomarcadores de alteração de proteínas reguladoras, por exemplo, medição dos níveis de proteínas de estresse, como as metalotioneínas;
- biomarcadores de disrupção endócrina e reprodutiva, com os quais se objetiva avaliar os níveis de estrogênio, anomalias no sistema reprodutor e capacidade de reprodução;
- biomarcadores de genotoxicidade, relativos à análise de alterações em nível da estrutura do DNA;
- biomarcadores de neurotoxicidade, que avaliam a atividade das colinesterases;
- biomarcadores de estresse oxidativo, que avaliam a atividade de enzimas antioxidantes, como por exemplo, da catalase, glutationa redutase e glutationa peroxidase;
- biomarcadores comportamentais, que avaliam categorias morfofuncionais de acordo com reações morfofisiológicas, tais como distúrbios respiratórios, distúrbios natatórios, distúrbios reprodutivos, etc.

Avaliações dos efeitos de contaminantes aos ecossistemas por meio da identificação de biomarcadores em cladóceros tem mostrado que estes estudos são adequados para um melhor entendimento das consequências de contaminação. Os biomarcadores mais utilizados nas avaliações ecotoxicológicas com cladóceros são: acetilcolinesterase (AChE) (Barata et al., 2001) e colinesterases em geral (ChE) (Damasio et al., 2007); catalase (CAT) (Kim et al., 2010); lactato desidrogenase (LDH) (Diamantino et al., 2001); monooxigenases como citocromo P 450-dependente (Sturm e Hansen, 1999); superóxido dismutase (SOD) (Barata et al. 2005); glutationa peroxidase (GPx) (Barata et al., 2005); glutationa S-transferase (GST) (Barata et al., 2005); peroxidação lipídica (LPO) (Barata et al., 2005) e enzimas digestivas (De Coen e Janssen, 1997). Segundo Barata et al. (2001), embora os cladóceros tenham sido amplamente estudados, o conhecimento relacionado às rotas metabólicas específicas é pouco conhecido. Os biomarcadores bioquímicos utilizados no presente estudo foram ChE, CAT e LPO e por isso, é discutido a seguir um pouco mais sobre eles.

Organismos aquáticos podem absorver e acumular compostos químicos em seus tecidos e órgãos em uma situação de contaminação. O metabolismo de detoxificação desses organismos age de forma a eliminar esses compostos, excretando-os. Para isso, ocorrem diversas reações catalisadas por enzimas que transformam esses contaminantes em compostos solúveis para serem mais facilmente excretados. A atividade de enzimas de biotransformação, de defesa

antioxidante e parâmetros de estresse oxidativo são exemplos de biomarcadores utilizados em avaliações da toxicidade e efeito de estressores sobre o organismo (Di Giulio e Hinton, 2008).

O metabolismo de biotransformação de compostos químicos pode ser dividido em três fases: na fase 1 ocorrem as reações de oxidação, redução ou hidrólise, na fase 2 acontecem as reações de conjugação e na fase 3 ocorre a excreção (Di Giulio e Hinton, 2008). O metabolismo de biotransformação, juntamente com o aumento do consumo de oxigênio para produção de energia na mitocôndria e ao aumento de reações redox promovidas por diversos tipos de compostos tóxicos nas células, gera espécies reativas de oxigênio (ERO) (*Reactive Oxygen Species*, ROS). Espécies reativas de oxigênio são, na maioria das vezes, radicais livres que podem reagir rapidamente com as biomoléculas causando sua desestruturação, muitas vezes com decorrente perda de sua função. Segundo Constantini (2010), alguns exemplos de ROS são os radicais superóxido e hidroxil, e outras moléculas pró-oxidantes, como o peróxido de hidrogênio.

As espécies reativas de oxigênio geradas no metabolismo de biotransformação são balanceadas pelas defesas antioxidantes como as Glutationas Peroxidases (GPx), Glutationa Redutase (GR), Catalase (CAT) e Superóxido Dismutase (SOD). Essas enzimas formam um sistema de defesa antioxidante que combate a formação das ERO convertendo os radicais reativos em moléculas não reativas (Van Der Oost et al., 2003). Um desequilíbrio entre a produção de enzimas reativas de oxigênio e a atuação de enzimas antioxidantes pode causar danos ao DNA, às proteínas e aos lipídeos, podendo afetar diretamente a permeabilidade das membranas celulares pelo processo de peroxidação lipídica.

A enzima catalase (CAT) é encontrada nas células animais, principalmente no fígado, rins e eritrócitos, estando presentes tanto no citoplasma como nas mitocôndrias (Lackner, 2008). Para a atividade da enzima, o ferro e a vitamina E são importantes coadjuvantes. Sua estrutura molecular é constituída por quatro subunidades, cada uma com um agrupamento porfirina (FeIII-protoporfirina) e uma molécula de NADPH (Hermes-Lima, 2004). A catalase apresenta um duplo papel, podendo funcionar como um catalisador na dismutação de moléculas de H_2O_2 e realizar a peroxidação quando se comporta apenas como aceptora de elétrons (Ahmad, 1995). A elevada atividade da catalase normalmente está relacionada com uma alta proliferação de peroxissomos e acredita-se que as ERO não induzam, por si só, elevadas atividades desta enzima. Dessa forma, a proliferação de peroxissomos aumenta a concentração de espécies reativas de oxigênio.

Quando algumas espécies reativas de oxigênio (ERO) apresentam reatividade suficiente para extrair um átomo de hidrogênio de um lipídeo intacto, inicia-se a lipoperoxidação ou peroxidação lipídica (LPO) (Filipak Neto, 2014), biomarcador bioquímico utilizado na determinação de estresse oxidativo, o qual tem início quando um intermediário reativo remove um hidrogênio de um ácido graxo poli-insaturado (PUFA). O radical lipídico formado pode reagir com oxigênio (O_2), produzindo um radical de peróxido lipídico, esta reação pode ser revertida e o oxigênio liberado, mas caso o PUFA contenha mais de três instaurações, o radical formado reage com outro radical de peróxido lipídico e forma um peróxido cíclico. Este radical pode reagir com outro ácido graxo, produzindo outro radical lipídico e um peróxido lipídico, fase denominada de propagação. Esse peróxido pode gerar radicais de alcóxido lipídico, podendo ser fragmentado em aldeídos lipídicos e outros radicais. Assim, como foi gerado um novo radical lipídico, esse ciclo de reações se propaga e causa danos à membranas, ácidos nucléicos, proteínas e outras funções até a fase de terminação. Nesta etapa, o sistema de defesa antioxidante celular começa a atuar, reagindo moléculas antioxidantes com os radicais peróxido e impedindo a produção de mais produtos que causam dano (Smart e Hodgson, 2008; Filipak Neto, 2014).

As colinesterases (ChE) pertencem à família de enzimas designadas como esterases, com a capacidade de hidrolisar ésteres carboxílicos. As ChE podem ser distinguidas das outras esterases, uma vez que exibem preferência pela hidrólise de ésteres de colina, em vez de outros ésteres carboxílicos (Gagne, 2014). A acetilcolinesterase (AChE) desempenha uma importante função no mecanismo de neurotransmissão, uma vez que promove a clivagem do neurotransmissor acetilcolina após sua liberação nas fendas sinápticas durante a transmissão colinérgica. A acetilcolina (ACh) é possivelmente a substância neurotransmissora mais bem conhecida nas sinapses químicas.

Em estudos ecotoxicológicos normalmente é proposta a utilização de uma série de biomarcadores, pois a avaliação de uma única resposta biológica pode não refletir de forma abrangente e complementar os danos na saúde dos organismos vivos de determinado ambiente impactado (Zorita et al., 2008). O acréscimo do uso em conjunto de diferentes biomarcadores é fundamental para minimizar interpretações errôneas nos casos de situações complexas de poluição (Pacheco e Santos, 2002).

Uma condição de estresse desencadeia a resposta de um organismo a um determinado tóxico/contaminante (De Coen et al., 2000). A resposta dos organismos a uma condição de estresse passa por diferentes fases referentes ao grau do distúrbio na função biológica normal e

o comprometimento do estado de saúde do organismo (Depledge et al., 1993) (Figura 6). Para manter suas funções vitais, o organismo saudável utiliza mecanismos homeostáticos em um estado de equilíbrio e dentro de uma faixa de funcionamento. Porém, sobre o efeito de um estressor, o organismo passa por uma perturbação inicial que causa respostas suborganismais, no qual, tentam fazer com que ele volte para um estado de equilíbrio (fase de compensação). Se o distúrbio aumenta, desvios a partir da condição saudável ocorrem e respostas compensatórias acontecem. Caso o distúrbio não seja compensado, a sobrevivência e reprodução são afetadas. No caso de o distúrbio persistir, o organismo morre, caracterizando a fase de não compensação, sendo incurável.

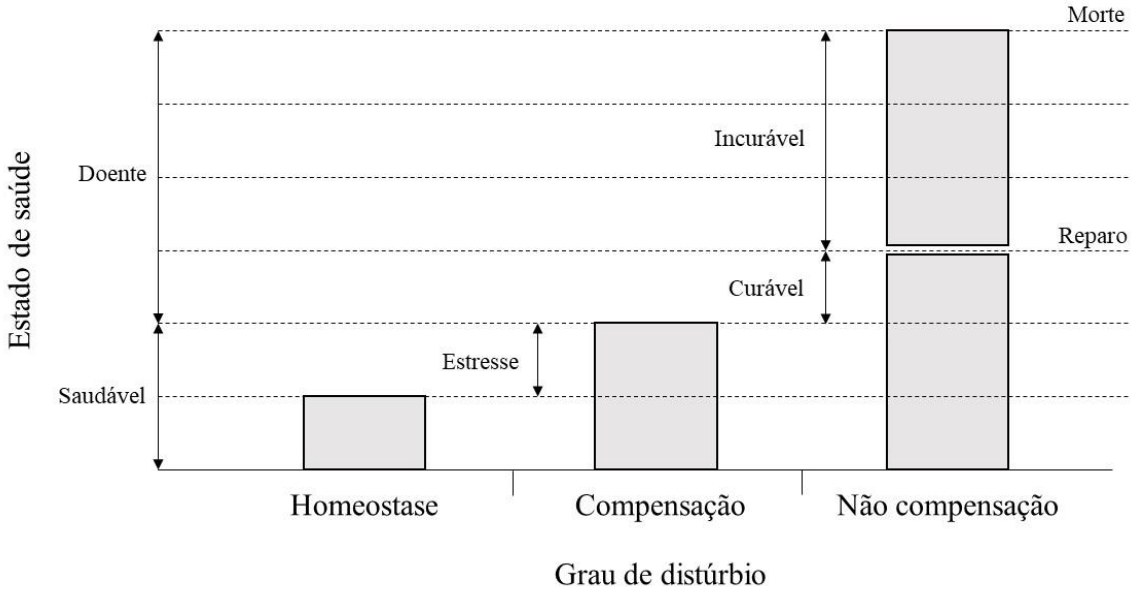

Figura 6. Representação de distúrbio induzido por estresse e subsequente dano a saúde. Fonte: Adaptado de De Coen et al. (2000) e Depledge (1989).

Biomarcadores bioquímicos medem as respostas biológicas iniciais causadas pela interação entre o compostoz/contaminante e o receptor-alvo biológico. Consequentemente, uma cascata de processos pode ter início, ou seja, uma resposta se inicia em nível molecular e repercute em níveis mais elevados de organização biológica (De Coen et al., 2000) (Figura 7). Nesta abordagem, reconhece-se que o distúrbio interfere em nível bioquímico e, por último, causa efeitos em nível de organismo (Coelho et al., 2011). Com o aumento dos níveis de organização biológica, os efeitos do distúrbio são traduzidos em parâmetros que não existem para um nível de organização inferior.

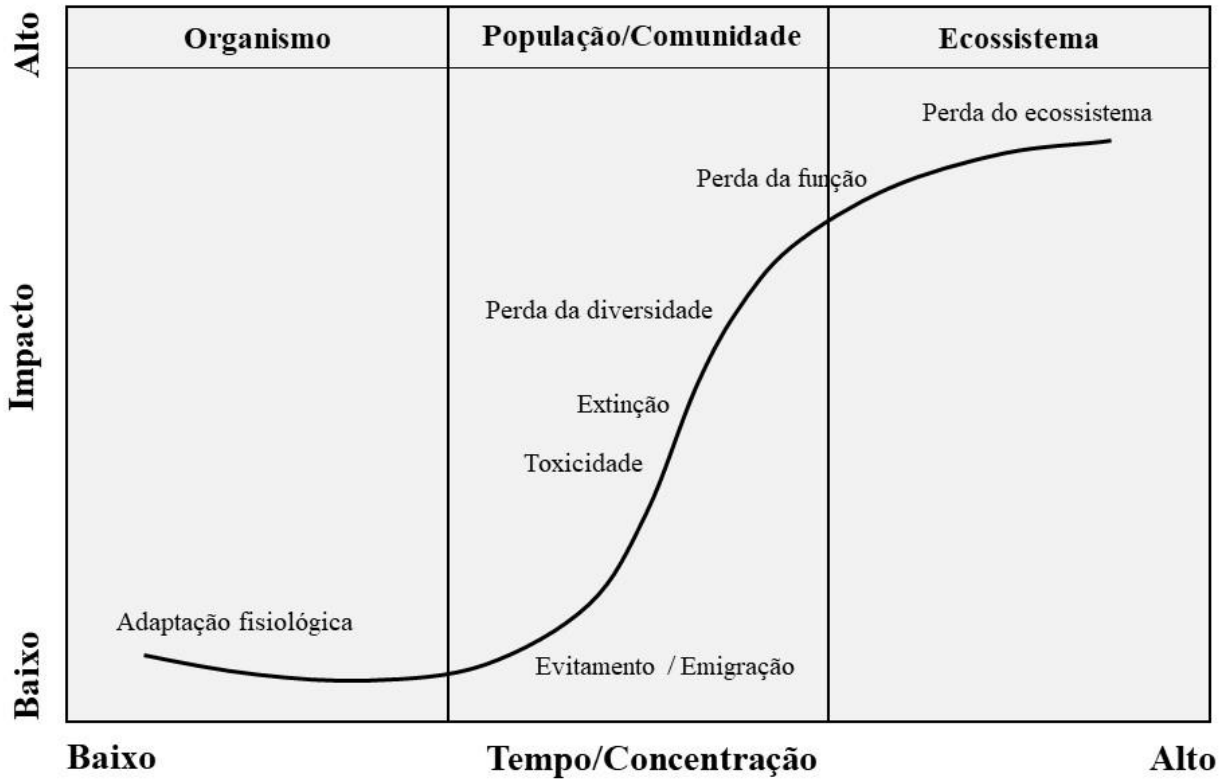

Figura 7. Representação de impactos do aumento do distúrbio induzido, que se inicia em nível molecular e repercute em níveis mais elevados de organização biológica. Fonte: De Coen et al. (2000).

1.7. Modelos ecossistêmicos (micro e mesocosmos)

A utilização dos testes de toxicidade em sistemas complexos (micro e mesocosmos) tem sido proposta como uma alternativa ou complementação na avaliação de risco ecológico, pois oferece resultados ecologicamente relevantes, que podem ser utilizados para validar e aperfeiçoar modelos teóricos (Boyle e Fairchild, 1997; Caquet; Lagadic e Steven, 2000). Modelos ecossistêmicos (micro e mesocosmos) são ecossistemas experimentais feito pelo homem caracterizados por juntar compartimentos ambientais naturais (água, sedimento e biota). Nesse sentido, os microcosmos aquáticos representam uma tentativa de melhor simular e prever os efeitos ambientais dos estressores, especialmente contaminantes, sobre a estrutura e função dos ambientes aquáticos (Bejarano et al., 2005) permitindo assim, o estudo dos efeitos de contaminantes sobre os organismos e comunidades sob condições semi-naturais, simuladas e controladas. Ou seja, micro/mesocosmos fazem a ligação entre a reprodutibilidade experimental e o realismo ecológico (Masutti, 2004) (Figura 8), fornecendo uma ponte entre o laboratório e o campo em termos de gerenciabilidade e permitindo a replicação e, portanto, uma

configuração experimental de um lado e proporcionando realismo em termos de processos ecológicos e exposições ao produto químico do outro lado.

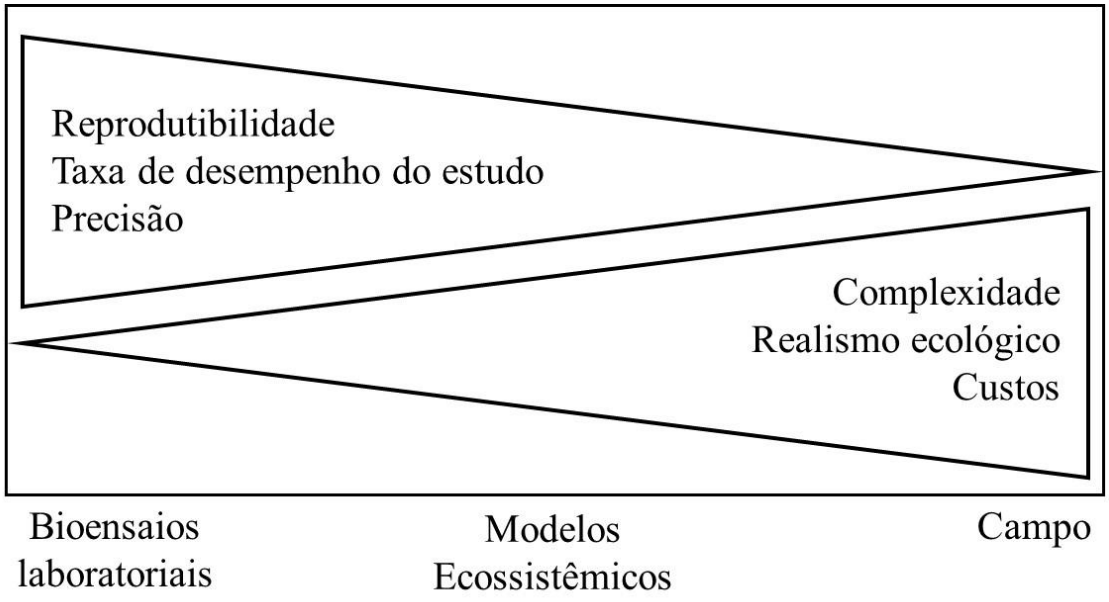

Figura 8. Representação de ecossistemas experimentais como ponte para os estudos de campo. Fonte: Adaptado de Brock et al. (2000).

Estudos em microcosmos ou mesocosmos contaminados com agrotóxicos vêm sendo utilizados em diferentes países devido à necessidade e importância das "validações de campo" dos resultados obtidos em testes de toxicidade realizados em laboratório, como por exemplo os estudos de Peither et al. (1996) realizados na Alemanha; Friberg-Jensen et al. (2003) na Dinamarca; Schroer et al. (2004) na Holanda; Chang; Sakamoto e Hanazato (2005) no Japão; López Mancisidor et al. (2008) na Espanha; Downing et al. (2008) nos Estados Unidos, Pablo e Hyne (2009) na Austrália e Novelli (2012); Moreira et al. (2017) no Brasil. Porém, em regiões tropicais, ainda são relativamente escassos estes estudos, provavelmente devido à complexidade, custo, tamanho da equipe e infraestrutura necessária para a realização dos mesmos.

Estudos ecotoxicológicos por meio de modelos ecossistêmicos são considerados como simulações do ambiente natural em escala reduzida, no qual, permite a análise dos efeitos de contaminantes em indivíduos, populações e comunidades sob condições seminaturais, possibilitando assim, o reconhecimento dos efeitos diretos e as implicações ecológicas indiretas (Graney, et al., 1995). Nesse caso, o estudo das interações entre as espécies presentes, por meio das análises dos efeitos diretos e indiretos dos contaminantes na comunidade e nos

compartimentos, tem dado suporte a compreensão da modulação e propagação dos efeitos de contaminantes no ambiente (Clements e Rohr, 2009).

A contaminação ambiental afeta o indivíduo, as populações, as comunidades e o ecossistema por meio de uma complexa interação entre os compartimentos e os organismos, a qual dificilmente poderia ser replicada em laboratório. Por isso, reconhece-se que os ecossistemas naturais apresentam características intrínsecas que influenciam o comportamento dos poluentes e, consequentemente, as respostas das diferentes espécies após um evento de contaminação. Dessa forma, estudos realizados em condições mais realísticas, como é o caso dos modelos ecossistêmicos, são mais efetivos na compreensão dos diferentes processos que ocorrem no ecossistema após adição de um contaminante estressor.

A maior parte dos estudos ecotoxicológicos, principalmente os que se referem aos micro e mesocosmos direcionados à avaliação dos riscos associados à contaminação ambiental por agrotóxicos, está centralizado em países de clima temperado e grande parte das informações geradas nessas condições tem servido como base para a formulação de políticas públicas e regulamentações ambientais em países da região Neotropical. Porém, as diferenças em relação aos parâmetros abióticos e bióticos, além da necessidade de preservação e conservação da biodiversidade tropical e vida silvestre (pois cerca de 75% da biodiversidade mundial está localizada nas regiões tropicais e subtropicais) demonstram a necessidade do desenvolvimento de estudos que tenham como objetivo a análise da distribuição da sensibilidade das espécies, o melhor entendimento do comportamento dos contaminantes em regiões de clima tropical, com intuito de proteger os ecossistemas naturais (terrestres, aquáticos e sua interface) e redução dos riscos inerentes à saúde humana (Kwok et al., 2007; Moreira et al., 2010).

Como a caracterização de um micro/mesocosmos é dependente de seu tamanho, o volume de água usado nos recipientes experimentais tem sido levado em consideração para caracterizar esses modelos ecossistêmicos. A Setac (*Society of Environmental Toxicology and Chemistry*) sugere o termo mesocosmos para represamentos artificiais maiores que 15m^3 (Caquet, 1989). Outros autores propõem volumes entre 1 e 10 m^3 (Lalli, 1990), 1 a 300 m^3 (La Point et al., 1989), 0,1 e 1000 m^3 (Graney et al., 1995). Contudo, o termo micro e mesocosmos, segundo Lalli (1990), pode ser usado para caracterizar os sistemas artificiais sob condições ambientais naturais com suficiente complexidade e estabilidade, em que o sistema se apresenta multitrófico e com tamanho suficiente para ser possível constatar alterações e relações dos contaminantes sobre a estrutura e dinâmica de funcionamento do ecossistema.

1.8. Justificativa

Grandes quantidades de substâncias químicas tóxicas são liberadas para o ambiente, contaminando o ar, solo e água (Bonaventura e Johnson, 1997). Entre os vários compostos que afetam os organismos de água doce, os agrotóxicos têm efeitos particularmente graves sobre a biota aquática, podendo ocasionar grandes perdas de biodiversidade (Aguilar-Alberola e Mesquita-Joanes, 2012). As condições geoclimáticas de cada ecossistema influenciam diretamente o efeito tóxico dessas substâncias aos organismos não-alvo. Dessa forma, avaliações de riscos ambientais gerados por agrotóxicos em condições tropicais são essencialmente recomendadas (Daam e Van den Brink, 2009), já que nos trópicos devido à mobilidade relativamente livre no solo e às chuvas torrenciais frequentes no verão, muitos agrotóxicos aplicados às culturas são lixiviados e, consequentemente, resíduos destes agrotóxicos podem ser encontrados em águas superficiais e subterrâneas (Wightwick Allinson, 2007; Pinheiro et al., 2010). Além disso, fatores como a qualidade do solo, que interfere na mobilidade dos compostos, temperaturas mais elevadas, que aumentam a solubilidade dos produtos em água e a absorção por organismos não-alvo, intensificam os riscos ambientais (Sanchez-Bayo e Hyne, 2011). Um exemplo desse cenário, é encontrado no município de Bom Repouso, localizado no sul do estado de Minas Gerais, o qual além de apresentar tais características ambientais, caracteriza-se pela existência de intensa atividade agrícola, destacando-se a produção das culturas de batata inglesa e morango.

O delineamento experimental deste estudo foi baseado no cenário descrito para o município de Bom Repouso (MG) e também associado ao fato de que existem poucas informações na literatura a respeito do potencial risco (toxicidade) destes agrotóxicos a espécies não-alvo em ecossistemas aquáticos tropicais, tanto agindo isoladamente, como em misturas. Com o intuito de maximizar a produção dessas culturas, diferentes tipos de agrotóxicos são aplicados, dentre eles o inseticida Kraft® 36 EC, cujo princípio ativo é a abamectina e o fungicida Score® 250 EC com o difenoconazol como princípio ativo, tendo esses contaminantes, grande potencial de atingir os ecossistemas aquáticos adjacentes às plantações.

Assim, faz-se necessário um estudo mais complexo da toxicidade dos agrotóxicos Kraft (abamectina) e Score (difenoconazol), o qual envolva invertebrados aquáticos não-alvo do modo de ação desses agrotóxicos e, aliado a isso, abordando diferentes níveis de organização biológica (molecular e individual) e estudos de organização complexa, em escala de microcosmos, que forneça uma melhor compreensão dos efeitos causados aos organismos da

biota aquática frente à contaminação por estes agrotóxicos, tanto isoladamente quanto em misturas.

O conhecimento da ligação entre as respostas dos biomarcadores bioquímicos e os efeitos adversos para níveis mais elevados de organização biológica é de grande importância, pois toda e qualquer modificação que aconteça em um processo fisiológico em decorrência da exposição a um dado composto tem relevância ecotoxicológica, e poderá ser utilizada em Toxicologia ambiental, pois será capaz de gerar informações relativas à natureza do processo toxicológico propriamente dito (identificação do orgão ou tecido alvo, por exemplo), da sua extensão e dos resultados esperados a partir dessa mesma exposição. Por esta razão, grande importância tem sido dada ao desenvolvimento de testes que objetivam a quantificação da atividade de enzimas que desempenham papeis cruciais em processos fisiológicos comuns, como a neutransmissão, a detoxificação, a respiração ou a resposta anti-oxidante. Consequências nestes níveis causarão provavelmente efeitos sobre a fisiologia dos organismos expostos, que poderão condicionar, posteriormente, alterações sobre a sobrevivência ou a reprodução, com possíveis consequências em nível populacional.

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2. Objetivos e hipóteses

2.1. Objetivo geral

Avaliar os efeitos tóxicos dos agrotóxicos Kraft® 36 EC (i.a. abamectina) e Score® 250 EC (i.a. difenoconazol), isolados e em mistura, sobre organismos zooplanctônicos.

2.2. Objetivos específicos

- Avaliar por meio de revisão bibliográfica a adequação de rotíferos para uso em avaliações de efeitos de agrotóxicos aos ecossistemas aquáticos, com especial ênfase para configurações tropicais;
- Analisar os efeitos sobre biomarcadores bioquímicos relacionados com atividades de neurotransmissão colinérgica (colinesterase - ChE) e relacionados com estresse oxidativo, catalase de enzimas antioxidantes (CAT) e peroxidação lipídica (LPO) na espécie *Daphnia magna* exposta aos compostos Kraft® 36 EC e Score® 250 EC isolados e em misturas;
- Avaliar parâmetros relacionados à energia (proteínas e lipídios totais) juntamente com o consumo de energia via sistema de transporte de elétrons no cladócero *Daphnia magna* exposto aos compostos Kraft® 36 EC e Score® 250 EC, isolados e em misturas;
- Determinar os efeitos da toxicidade aguda e crônica dos agrotóxicos Kraft® 36 EC (abamectina) e Score® 250 EC (difenoconazol) em ação individual e em misturas sobre parâmetro reprodução no cladócero *Daphnia magna*;
- Simular e avaliar os efeitos ambientais gerados em ecossistemas aquáticos tropicais por meio de testes de toxicidade com as diluições da água do escoamento superficial (*runoff)* contaminada pelos agrotóxicos Kraft® 36 EC e Score® 250 EC, isolados e em mistura, à espécie *Macrothrix flabelligera*, por meio de experimentos laboratoriais;
- Avaliar a toxicidade das amostras de água de microcosmos contaminados com escoamento de água superficial (*runoff)* e aplicação direta (*spray drift*) pelos agrotóxicos Kraft® 36 EC e Score® 250 EC, isolados e em mistura, assim como compará-las às respostas encontradas após testes de toxicidade aguda dos agrotóxicos supracitados, também isoladamente e em mistura à espécie *M. flabelligera*;

- Comparar a sensibilidade de diferentes organismos-teste pertencentes a diferentes grupos taxonômicos, por meio de curvas de distribuição da sensibilidade das espécies (SSD) e verificar as espécies mais sensíveis aos agrotóxicos testados.

2.3. Hipóteses

- Espécies do Filo Rotifera podem ser recomendadas para serem utilizadas na avaliação de risco e efeitos de agrotóxicos aos ecossistemas aquáticos tropicais.
- Os agrotóxicos Kraft® 36 EC (abamectina) e Score® 250 EC (difenoconazol), tanto isolados como em mistura, causarão efeitos deletérios aos organismos-teste;
- As misturas destes dois agrotóxicos tem sua toxicidade e seus efeitos deletérios potencializados, caracterizando um efeito sinérgico quando expostos a organismos aquáticos;
- O *runoff* contaminado com esses compostos causa efeito deletério (mortalidade ou imobilidade) a organismos-teste não-alvo;
- As alterações na atividade de enzimas de neurotransmissão e de estresse e dano oxidativo em *Daphnia magna* funcionarão como biomarcadores bioquímicos dos efeitos deletérios a este organismo-teste quando exposto aos agrotóxicos Kraft® 36 EC (abamectina) e Score® 250 EC (difenoconazol);
- Os agrotóxicos Kraft® 36 EC (abamectina) e Score® 250 EC (difenoconazol), tanto isolados como em mistura, causarão alterações em parâmetros relacionados à energia (proteínas e lipídios totais) e no consumo de energia via sistema de transporte de elétrons em *D. magna*.
- O cladócero nativo da região Neotropical, *Macrothrix flabelligera* é mais sensível do que espécies de região temperada recomendadas em protocolos padronizados para a avaliação da toxicidade dos agrotóxicos abamectina e difenozonazol.

Capítulo 1. The use of rotifers as test species in the aquatic effect assessment of pesticides in the tropics (Review Paper)

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Abstract

The present study aimed at evaluating the suitability of rotifers as standard invertebrate test species for the aquatic effect assessments of pesticides, with special emphasis to tropical settings. This was done by weighing rotifers against the criteria that are traditionally used for this end. Rotifers are easy to maintain and culture in the laboratory and their (biological) response to chemical stressors like pesticides is well known. As abundant organisms in aquatic ecosystems, they play a key role in energy flow and nutrient cycling. Although they are often considered to have a low sensitivity to pesticides, a sensitivity analysis conducted in this study revealed that they may be more sensitive than the standard invertebrate test species *Daphnia magna* to fungicides. In addition, few toxicity data were available for rotifers other than *Brachionus calyciflorus* and these data were almost exclusively acute (EC_{50}) toxicity values. Subsequently, the sensitivity of other rotifers as well as the chronic sensitivity, bioaccumulation potential and possible role in biomagnification of pesticides in aquatic foodwebs remains largely unknown. Given their greater diversity and ecological role in tropical freshwaters as compared to temperate freshwaters, the use of rotifers in tropical risk assessments and immediate research needs are discussed.

Keywords: rotifers; tropics; aquatic ecotoxicology; ecological risk assessment; pesticides

1. Introduction

Ecological risk assessments involve a comparison of the (predicted or measured) exposure level of a chemical with the probability to cause toxic effects at that level. Initial or lower-tier toxicity assessments are traditionally conducted by testing a concentration series of the test compound in single species tests as to establish toxicity threshold values (e.g. Concentration of half-maximal response - EC_{50}, No Observed Effect Concentration - NOEC).

These tests are conducted with a limited number of standard test species selected as surrogates for the sensitivity of other organisms that may exist in natural ecosystems. In Europe,

for example, the first-tier prospective effect assessment of pesticides for aquatic organisms in edge-of-field surface waters is based on toxicity tests using test-organisms representing different trophic levels, for which there are internationally standardized protocols, such as: the fish *Oncorhynchus mykiss*, algae *Pseudokirchneriella subcapitata*, the macrophyte *Lemna* spp. and the invertebrates. Regarding invertebrates, testing *Daphnia* sp. (preferably *D. magna*) is always required but additional invertebrate tests with the saltwater crustacean *Americamysis bahia*, the insect *Chironomus* sp. and/or the oligochaete *Lumbriculus* sp. may also be required depending on the type and mode-of-action of the pesticide of concern (*European Food Safety Authority* - EFSA, 2013). Single species tests with rotifers are hence not required in this first-tier effect assessment, although they may be included in higher-tier assessments (geomean and species sensitivity distributions approaches) and rotifers are generally also well-represented in model ecosystem studies (EFSA, 2013).

Ecotoxicological research into the fate and side-effects of agrochemicals on aquatic ecosystems surrounding agricultural fields has focused almost exclusively on temperate countries. Hence, aquatic risk assessments in tropical countries often rely on temperate toxicity data, even though the fate and effects of pesticides may be different between climatic regions (e.g. Lacher and Goldstein, 1997; Kwok et al., 2007; Daam and Van den Brink, 2010a). Given the enormous natural variability in the structure and function of freshwater communities, it is reasonable to question whether the set of standard test species generally used in temperate regions is appropriate for tropical ecosystems. For example, the standard invertebrate test species *D. magna* does not exist in tropical freshwater ecosystems. Subsequently, other invertebrate taxa well-represented in tropical edge-of-field waterbodies could be more suitable candidates as surrogates for invertebrate communities in tropical risk assessment schemes. In this regard, the need to develop indicator species belonging to the Phylum Rotifera for ecotoxicological studies in tropical waters has previously been expressed (e.g. Snell and Joaquim-Justo, 2007).

The aim of the present study was to evaluate the suitability of rotifers to serve as standard invertebrate test species in tropical aquatic effect assessments. This was done by weighing rotifers against the (adapted) criteria for the selection of suitable organisms in single-species toxicity testing by Rand et al. (1995) and Van Leeuwen (1995): they should 1) be easy to keep in the laboratory or can reproduce and be cultured under laboratory conditions; 2) have adequate background information on their biology and response to toxicity available as reference database; 3) be valuable in terms of economics, ecology (e.g. key organism) and/or

recreation; 4) be native to (or representative of) the ecosystem under study, and 5) be sensitive to a wide range of compounds. Each of these criteria is briefly evaluated in the following sections, followed by an overall conclusion on the use of rotifers as test organisms in tropical effect assessments based on the assessment of these criteria.

2. Ease to keep and culture in the laboratory

Numerous studies have indicated that the biological attributes of rotifers readily recommend them as test organisms (e.g. Snell and Janssen, 1995; Snell and Joaquim-Justo, 2007; Dahms et al., 2011). Their small size, high fecundity and short life cycle means that several rotifer species have been successfully grown in the laboratory (Snell and Joaquim-Justo, 2007). In addition, because of their breeding strategies and widespread dispersion in the form of resistant eggs or by zoochoric transport, rotifer enjoy great ecological success and are hence readily available (Ruppert and Barnes, 1994). Despite these traits, only two protocols have so far been standardized for toxicity tests performed with rotifers, namely the American Society for Testing and Materials - ASTM (1991) protocol for acute testing and the Snell (1998) protocol for chronic toxicity with the freshwater rotifer *B. calyciflorus* and the brackish-water species *B. plicatilis.*

3. Background information on biology and response to toxicity

The Phyllum Rotifera has about 2030 currently known species and is classified into three main groups: Seisonidae, Bdelloidea and Monogononta (Segers, 2007). These are small metazoans, unsegmented, with bilateral symmetry and generally between 100 and 1000 microns long, although, some species can reach 3000 micrometers (Edmondson, 1959). Their biology and taxonomy is generally well known and detailed identification keys have long been developed (e.g. Edmondson, 1959; Ruttner-Kolisko, 1974; Koste, 1978).

There has been an increasing number of studies with rotifers aiming to assess the effects of pesticides on their life-cycles and intrinsic population growth rates, as well as mortality, reproduction, delayed development, morphological alterations, amictic female production, resting egg production, probability of extinction, feeding, swimming activity, and in vivo enzyme activity (Table 1). Besides the great variety in responses to toxicity that have been evaluated in these studies, it has also been shown that the biological responses of rotifers to toxicants like pesticides are reproducible (Suga et al., 2007).

Table 1. Endpoints commonly analyzed when using rotifers as test-organisms for pesticide toxicity assessments.

Rotifer species	Pesticides (type - chemical class; common name)	Endpoints	References
Brachionus calyciflorus	Insecticide – Organochlorine: dieldrin	Amitic females production	Huang et al., 2013
Brachionus calyciflorus	Herbicide – Carbamate: molinate and thiobencarb	Intrinsic population growth rate	Ferrando et al., 1999
Brachionus calyciflorus	Fungicide – Dicarboximide: vinclozolin	Morphological alterations	Alvarado-Flores et al., 2015
Brachionus calyciflorus	Insecticide – Organochlorine: endosulfan	Mortality	Fernandez-Casalderrey et al., 1992
Brachionus calyciflorus	Insecticide – Organophosphate: chlorpyrifos	Reproduction	Snell and Carmona, 1994
Brachionus calyciflorus	Insecticide – Organophosphate: dimethoate	Swimming behavior	Guo et al., 2012
Brachionus plicatilis	Insecticide/fungicide – Organochlorine: pentachlorophenol	Enzime activity	Moffat and Snell, 1994
Brachionus plicatilis	Insecticide – Organophosphate: chlorpyrifos	Feeding	Juchelka and Snell, 1995
Brachionus plicatilis	Insecticide – Organophosphate: diazinon	Resting egg production	Marcial et al., 2007
Brachionus rubens	Insecticide/fungicide – Organochlorine: pentachlorophenol	Life-cycle	Halbach et al., 1983
Brachionus sp.	Insecticide - triazine	Delayed development	Rioboo et al., 2007
Lecane quadridentata	Insecticida – Carbamate: carbaryl Insecticida – Organophosphate: methyl parathion	Probability of extinction	Pérez-Legaspi et al., 2010
Asplanchna girodi *Euchlanis dilatata* *Keratella cochlearis* *Lecane quadridentata* *Lepadella patella* *Philodina acuticornis* *Plationus patulus* *Trichocerca pusilla*	Insecticide/fungicide – Organochlorine: pentachlorophenol	Mortality and Enzime activity	McDaniel and Snell, 1999

4. Valuable in terms of economics and/or ecology

As generalist organisms, rotifers consume a variety of food items in their natural habitats - debris, bacteria and algae - and are subject to significant predation (Barnes et al., 2001). They have a key role in energy flow and nutrient cycling in aquatic ecosystems, because they act with remarkable efficiency in the conversion of much of the primary production of algae and bacteria into low secondary consumers like insect larvae and fish (Nogrady et al., 1993). In Lake

Nakuru, for example, the rotifers *Brachionus dimidiatus* and *B. plicatilis*, even though not especially significant in terms of biomass, had the highest production rates (1.7 KJ m^{-3} d^{-1}) out of all invertebrates evaluated (Vareschi and Jacobs, 1984). Doohan (1973) presented an energy budget for adults of the brackish waters rotifer *Brachionus plicatilis*: At 20°C, the hourly consumption of *Dunaliella salina* by an individual rotifer was found to be 333 ± 93 cal, with a measured assimilation rate of 64 ± 10 cal hr^{-1}.

Despite their small size, they contribute significantly to secondary production in aquatic systems because of the large population sizes that can be attained due to their rapid growth, short life-cycle and, sometimes, parthenogenetic reproduction (Snell and Janssen, 1995; Wallace et al., 2006). For example, Rothhaupt (1990) showed that the rotifers *B. calyciflorus* and *B. rubens* had maximal growth rates slightly below 0.8 day^{-1}, a value that is comparable to that of most protozoan species. Since they are important prey organisms for fish and shrimps that may in turn be used for human consumption, rotifers are indirectly also important in economic terms.

5. Native to (or representative of) the ecosystem under study

According to Fernando (1994), the major difference in fish and zooplankton between tropical and temperate freshwaters is the predominance of Rotifera and herbivorous fish in tropical freshwater ecosystems *versus* Crustacea and non-herbivorous fish in their temperate counterparts (Figure 1). Although rotifers were historically considered as cosmopolitan, a study into the world´s distribution of rotifers by De Ridder (1981) indicated that although 52% are indeed cosmopolitan, the remaining 48% have a more or less limited distributional area of which 7% are endemic in some areas. In this regard, rotifer diversity has been reported to be highest in the (sub) tropics, with rotifer biodiversity hotspots in northeast North America, tropical South America, Southeast Asia, Australia, and Lake Baikal (Segers, 2008). However, as pointed out by Fontaneto et al. (2012), it should be taken into account that sampling intensity influences the results of diversity geographical distribution masking the actual distributional patterns, and that therefore this factor should be carefully considered before drawing conclusions from distributional analysis. Also, García-Morales and Elías-Gutiérrez (2013) called attention for the need of additional taxonomic molecular studies, such as DNA barcoding, throughout the world in order to understand the processes that drive global patterns of rotifer diversity.

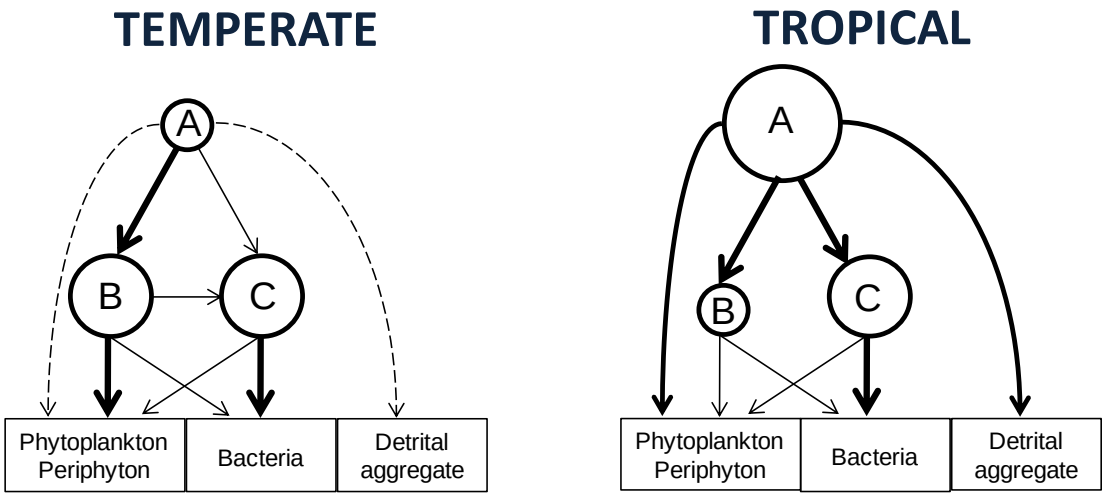

Figure 1. Main freshwater ecosystem components and their food interrelationships in temperate versus tropical freshwaters. Sizes of the circles indicate the relative size of the ecosystem components, the thickness of the arrow the strength of the interaction between them, while its direction points to the consumer - food relationship. Source modified from Fernando (1994) and Daam and Vanden Brink (2011).

Given the above, it may be deduced that rotifers and crustaceans are the dominant and keystone zooplanktonic invertebrates in tropical and temperate freshwaters, respectively. In addition, due to year-round predation by the great diversity of fish and invertebrate predators and increased metabolic costs with increasing temperatures, large cladocerans like *Daphnia* have been reported to be practically absent in the tropics (e.g. Fernando, 1994; Dumont, 1994). The ecological relevance of using toxicity data derived from tests with daphnids in tropical aquatic risk assessments is therefore highly disputable (Daam and Van den Brink, 2010b; Allinson et al., 2011).

6. Sensitivity

Rotifers have been shown to be among sensitive primary consumers, more sensitive to some chemicals than cladocerans (Snell and Joaquim-Justo, 2007; Dahms et al., 2011). On the other hand, rotifers are generally considered to have low sensitivity to pesticides and have frequently shown increased abundances in temperate model ecosystem studies evaluating insecticides as a result of decreased competition for food through the death of sensitive crustacean taxa (e.g. Brock et al., 2000; Hanazato, 2001). To evaluate this further, freshwater laboratory toxicity data for rotifers were compiled from the *United States Environmental Protection Agency* - US-EPA ECOTOX database (http://cfpub.epa.gov/ecotox/) and compared

with those for *D. magna* originating from the same source. Only data for compounds classified in the Alan Wood Compendium (http://www.alanwood.net/pesticides/) as insecticides or fungicides were analyzed further since a greater sensitivity of rotifers may be expected for these pesticide types. To allow comparison of toxicity data between taxa, only data fulfilling the selection criteria provided in Table 2 were used. The geometric mean was calculated if more than one toxicity value was available for a given species and compound. Rotifer short-term toxicity data were obtained for 12 insecticides, but only almost exclusively for *B. calyciflorus* (see Supplementary Material Table S1). Besides carbaryl, for which the toxicity value of *B. calyciflorus* was approximately 5 times lower than that of *D. magna*, the latter species was one to four orders of magnitude more sensitive than *B. calyciflorus*.

Table 2. Selection criteria for short-term and long-term toxicity test data (adapted from Van den Brink et al., 2006 and EC, 2011).

	Short-term toxicity tests	**Long-term toxicity tests**
Endpoint	EC50; LC50	NOEC; EC10; EC10-20/2; MATC/$\sqrt{2}$
Test parameters	Mortality, immobilization	Growth, feeding, reproduction, development, mortality or immobilization
Test duration (d)	1 - 7	> 2

Short-term fungicide data (see Supplementary Material Table S2), however, indicated that *B. calyciflorus* had similar toxicity to copper sulfate, greater sensitivity to pentachlorophenol (PCP; 2 times) and fluazinam (113 times), although it appears eight times less sensitive to vinclozolin than *D. magna*. The few fungicide toxicity data obtained for rotifer taxa other than *B. calyciflorus* showed a similar pattern. *Brachionus rubens* showed a similar sensitivity as *D. magna* (and *B. calyciflorus*) to copper sulphate, whereas *Philodina acuticornis* was 60 times less sensitive to this compound but 33 more sensitive to chlorothalonil than *D. magna* (no data available for *B. calyciflorus*). Most rotifer toxicity data was obtained for PCP, and the sensitivity distribution of rotifer taxa and *D. magna* for this compound is provided in Figure 2. *D. magna* as well as *B. calyciflorus* appear relatively tolerant to this compound, with several rotifers showing an up to two orders of magnitude greater sensitivity (Figure 2). The suitability of the commonly tested *B. calyciflorus* as sole test species for the sensibility of all rotifers has indeed often been disputed (e.g. McDaniel and Snell, 1999; Wallace et al., 2006; Moreira et al., 2015). Locally available taxa other than *B. calyciflorus* are seldom considered but may show a greater sensitivity to chemical stress (e.g. Gama Flores et al., 2004). In line with this, the tropical rotifer *Keratella tropica* was noted to be the most sensitive zooplankton

species to carbendazim in an outdoor model ecosystem study conducted in Thailand (Daam et al., 2010). In the same study, *B. calyciflorus* appeared to be amongst the most tolerant zooplankton taxa and increased in numbers even when exposed to the highest carbendazim concentration tested (Daam et al., 2010). These authors further noted that the temperate species *K. quadrata* was among the most affected zooplankton taxa in model ecosystem studies evaluating carbendazim conducted by Slijkerman et al. (2004) and Van den Brink et al. (2000).

In a microcosm study evaluating the fungicide triphenyltin acetate, *K. quadrata* was also the most sensitive zooplankton taxon and showed a quick negative response to the fungicide (Roessink et al., 2006). Another *Keratella* species, *K. cochlearis*, was the most sensitive taxon of six rotifer taxa tested to the fungicide pentachlorophenol as determined by swimming behavior and vulnerability to predation (Preston et al., 1999). Apparently, the genus *Keratella* includes the most susceptible representatives of rotifers and often even the zooplankton community to fungicides (Daam et al., 2010).

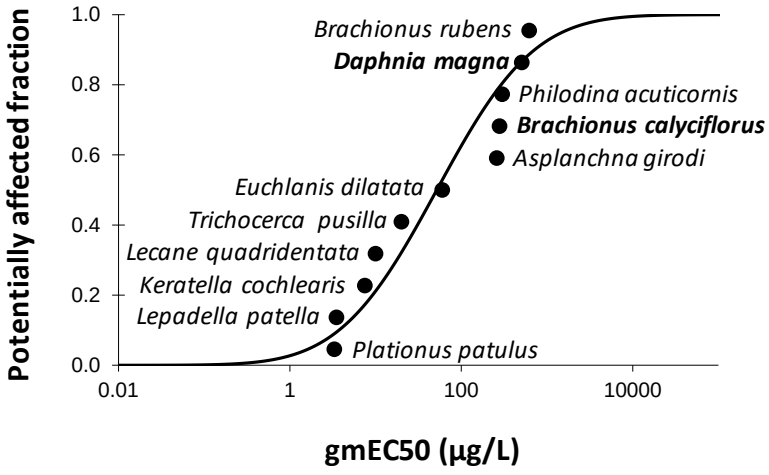

Figure 2. Species sensitivity distribution constructed with EC_{50} data for pentachlorophenol (PCP) of rotifers and *D. magna*.

The greater diversity and limited distribution of rotifers in the tropics as discussed in the previous section also implies that the number of potential sensitive species that could be present and hence affected by pollutants is also greater in tropical freshwaters. In addition, several studies have shown that rotifer taxa previously considered to be cosmopolitan can be complexes of sibling species, including *B. calyciflorus* (e.g. Gilbert and Walsh, 2005). Different strains of the same species as well as experimental conditions (e.g. temperature) may influence sensitivity

levels of rotifers (e.g. Serrano et al., 1986; Snell et al., 1991). Subsequently, it is imperative to test locally-derived rotifers in tropical toxicity tests to obtain an accurate assessment of their true sensitivity (Moreira et al., 2015).

The toxicity values for rotifers available in the US-EPA database were almost exclusively short-term EC_{50} values. Besides NOEC values of the insecticide lindane for *Brachionus angularis* and *Brachionus rubens*, NOEC values were exclusively encountered for *B. calyciflorus* and only for five insecticides and two fungicides (see Supplementary Material Tables S3 and S4, respectively). This limited availability of chronic rotifer toxicity data has previously been noted by Preston and Snell (2001) and Marcial et al. (2005). These authors discussed that because only a portion of the rotifer life cycle is investigated in the acute EC_{50} rotifer toxicity tests, the true vulnerability of rotifer life cycles to toxicants is often underestimated. Especially fertilization appears to be a sensitive chronic endpoint, as demonstrated by Preston et al. (2000) for a number of potential endocrine disruptors including pesticides. Since pesticides are generally applied with a high frequency in the tropics (Daam and Van den Brink, 2010; Sanchez-Bayo and Hyne, 2011), a chronic (pulsed) exposure and hence chronic toxicity tests may be especially relevant for tropical toxicity testing.

Besides a limited availability of chronic toxicity data, data on the bioconcentration and bioaccumulation potential of insecticides and fungicides were completely lacking in the US-EPA database. Given the prominent role that rotifers play in tropical aquatic foodwebs (Figure 1), bioconcentration and bioaccumulation studies are relevant to assess the potential of pesticide biomagnification by rotifers. Sarma et al. (1998), for example showed that rotifer prey (*B. calyciflorus*) exposed to sublethal methyl parathion concentrations for 2 hours had a significant negative effect on the population growth of its predator (*Asplanchna sieboldi*). The great importance of the trophic pathway (food ingestion) and the great potential of pollutants to accumulate through the aquatic trophic food chain (algae, zooplankton, fish) has previously been demonstrated for metals (e.g. Dobbs et al., 1996; Alvarado-Flores, 2012) and polychlorinated biphenyls - PCBs (e.g. Joaquim-Justo et al., 1995). For exemple, exposure to lead resulted in deposited granules of this metal in the mastax and vitellarium of *B. calyciflorus* (Alvarado-Flores, 2012), and *Lecane quadridentata* showed adverse effects on growth rate after lead bioaccumulation (Hernández-Flores and Rico-Martínez, 2006).

7. General remarks and conclusions

For the reasons discussed above, the use of rotifers as test organisms in ecotoxicology has been recognized for some time (e.g. Snell and Janssen, 1995; Gama-Flores et al., 2004; Snell and Joaquim-Justo, 2007). US-EPA (2001) also stressed the need for conducting more studies with rotifer taxa other than *B. calyciflorus* to allow comparing the intra-and inter-species differences in the sensitivities of rotifers to environmental pollutants. Besides acute toxicity testing, such studies should also include chronic evaluations and studies into bioaccumulation and biomagnification.

From the above, it may also be concluded that given their role and diversity in tropical freshwater ecosystems, this great potential of rotifers as test species holds especially true for the tropics (e.g. Snell and Joaquim-Justo, 2007). In a review paper by Organization for Economic Co-operation and Development - OECD as far back as 1998, rotifers were already identified as important test species to be included in warm freshwater environments. Future research efforts should shed light on i) the acute and chronic sensitivity of local taxa; ii) the bioaccumulation potential and possible role in biomagnification through the aquatic foodweb; iii) candidates to be used as tropical test species surrogates for *B. calyciflorus*.

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Supplementary material

Table S 1. Short-term (EC_{50} and LC_{50}) rotifer toxicity data for insecticides as compiled from the US-EPA ECOTOX database (http://cfpub.epa.gov/ecotox/).

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
Carbaryl	*Brachionus calyciflorus*	1	Mortality	4100	9
Carbofuran	*Brachionus calyciflorus*	2	Abundance	1670	2
Carbofuran	*Brachionus calyciflorus*	1	Abundance	2000	2
Chlorpyrifos	*Brachionus calyciflorus*	1	Mortality	11850	5
Chlorpyrifos	*Brachionus calyciflorus*	1	Mortality	11900	14
Chlorpyrifos	*Brachionus calyciflorus*	2	Mortality	12000	14
Chlorpyrifos	*Brachionus calyciflorus*	2	Mortality	12000	15
Chlorpyrifos	*Brachionus calyciflorus*	1	Mortality	12000	17
Diazinon	*Brachionus calyciflorus*	1	Mortality	29220	3
Diazinon	*Brachionus calyciflorus*	1	Mortality	29220	4
Diazinon	*Brachionus calyciflorus*	2	Mortality	31000	14
Diazinon	*Brachionus calyciflorus*	2	Mortality	31000	15
Endosulfan	*Brachionus calyciflorus*	1	Mortality	5150	3
Endosulfan	*Brachionus calyciflorus*	1	Mortality	5150	37
Endosulfan	*Brachionus calyciflorus*	1	Mortality	5150	37
Endosulfan	*Brachionus calyciflorus*	1	Mortality	5150	6
Fenitrothion	*Brachionus calyciflorus*	1	Mortality	6680	5
Fenitrothion	*Brachionus calyciflorus*	1	Mortality	6700	14

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
Fenitrothion	*Brachionus calyciflorus*	1	Mortality	6700	17
Fenitrothion	*Brachionus sp.*	2	Mortality	3980	8
Fenitrothion	*Brachionus sp.*	1	Mortality	6300	8
Fenthion	*Brachionus sp.*	2	Mortality	5620	8
Fenthion	*Brachionus sp.*	1	Mortality	7240	8
Lindane	*Brachionus calyciflorus*	1	Mortality	7660	1
Lindane	*Brachionus calyciflorus*	1	Mortality	22500	5
Lindane	*Brachionus calyciflorus*	1	Mortality	22500000	6
Malathion	*Brachionus calyciflorus*	2	Abundance	1950	2
Malathion	*Brachionus calyciflorus*	1	Abundance	2660	2
Malathion	*Brachionus calyciflorus*	1	Mortality	33720	4
Malathion	*Brachionus calyciflorus*	1	Mortality	80840	10
Malathion	*Brachionus calyciflorus*	1	Mortality	80840	11
Malathion	*Brachionus rubens*	1	Mortality	35300	16
Methyl parathion	*Brachionus angularis*	1	Mortality	636	7
Methyl parathion	*Brachionus angularis*	1	Mortality	2783	7
Methyl parathion	*Brachionus angularis*	1	Mortality	6520	7
Methyl parathion	*Brachionus calyciflorus*	1	Mortality	29190	4
Methyl parathion	*Brachionus patulus*	1	Mortality	8850	13
Methyl parathion	*Brachionus patulus*	1	Mortality	10660	13
Phosphamidon	*Brachionus sp.*	2	Mortality	360	8
Phosphamidon	*Brachionus sp.*	1	Mortality	3800	8

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
Trichlorfon	*Brachionus calyciflorus*	1	Mortality	51940	5
Trichlorfon	*Brachionus calyciflorus*	1	Mortality	46600	14
Trichlorfon	*Brachionus calyciflorus*	1	Mortality	47000	17
Trichlorfon	*Filinia longiseta*	4	Mortality	14	12
Trichlorfon	*Keratella quadrata*	4	Mortality	16	12

Table S 2. Chronic rotifer toxicity data for insecticides as compiled from the US-EPA ECOTOX database (http://cfpub.epa.gov/ecotox/).

Pesticide	Species scientific name	Test duration (days)	Endpoint	Effect	Concentration (µg/L)	Reference no.
Carbaryl	*Brachionus calyciflorus*	2	NOEC	Intrinsic rate of increase	540	9
Carbaryl	*Brachionus calyciflorus*	7	NOEC	Progeny	60	9
chlorpyrifos	*Brachionus calyciflorus*	2	MATC	Population Growth Rate	270	14
chlorpyrifos	*Brachionus calyciflorus*	2	NOEC	Population Growth Rate	230	14
chlorpyrifos	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	230	15
chlorpyrifos	*Brachionus calyciflorus*	2	MATC	Reproduction, general	240	15
chlorpyrifos	*Brachionus calyciflorus*	2	MATC	Reproduction, general	350	15
chlorpyrifos	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	200	15
chlorpyrifos	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	300	15
chlorpyrifos	*Brachionus calyciflorus*	3	NOEC	Pregnant females in a population	100	18
chlorpyrifos	*Brachionus calyciflorus*	4	NOEC	Fertilization	10	19

Diazinon	*Brachionus calyciflorus*	2	MATC	Population Growth Rate	10000	14
Diazinon	*Brachionus calyciflorus*	2	NOEC	Population Growth Rate	8000	14
Diazinon	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	8000	15
Endosulfan	*Brachionus calyciflorus*	3	NOEC	Population Growth Rate	3500	21
Endosulfan	*Brachionus calyciflorus*	3	NOEC	Fertility	875	21
Endosulfan	*Brachionus calyciflorus*	3	NOEC	Pregnant females in a population	3500	21
Lindane	*Brachionus angularis*	28	NOEC	Mortality	12	20
Lindane	*Brachionus rubens*	28	NOEC	Mortality	55	20

Table S 3. Short-term (EC_{50} and LC_{50}) rotifer toxicity data for fungicides as compiled from the US-EPA ECOTOX database (http://cfpub.epa.gov/ecotox/).

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
Chlorothalonil	*Philodina acuticornis odiosa*	1	Mortality	3.2	26
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	200	25
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	76	6
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	380	6
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	16,6	10
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	26	29
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	33	29
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	33	29
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	38	29
Copper sulfate (anhydrous)	*Brachionus calyciflorus*	1	Mortality	9,4	30
Copper sulfate (anhydrous)	*Brachionus rubens*	1	Mortality	19	16
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Immobilization	140	22
Copper sulfate (anhydrous)	*Philodina acuticornis*	4	Immobilization	160	22
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Immobilization	220	22
Copper sulfate (anhydrous)	*Philodina acuticornis*	4	Immobilization	600	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	4	Immobilization	700	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Immobilization	800	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Immobilization	1000	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	4	Immobilization	1100	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	4	Immobilization	1100	23

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Immobilization	1500	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Immobilization	1900	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Immobilization	5400	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Immobilization	5800	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Immobilization	5800	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Immobilization	6400	23
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Mortality	950	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Mortality	1000	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Mortality	1130	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Mortality	1150	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Mortality	1200	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Mortality	1210	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Mortality	1300	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	2	Mortality	1300	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Mortality	1430	24
Copper sulfate (anhydrous)	*Philodina acuticornis*	1	Mortality	1500	24
Fluazinam	*Brachionus calyciflorus*	2	Mortality	1.6	33
PCP (sodium salt)	*Asplanchna girodi*	1	Survivorship	260	28
PCP (sodium salt)	*Brachionus calyciflorus*	6	Mortality	200	27
PCP (sodium salt)	*Brachionus calyciflorus*	2	Mortality	400	27
PCP (sodium salt)	*Brachionus calyciflorus*	1	Survivorship	160	28
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	82	31
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	82	31

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	83	31
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	91	31
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	95	31
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	170	31
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	210	31
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	262	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	362	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	378	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	450	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	567	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	714	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	738	32
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	1200	14
PCP (sodium salt)	*Brachionus calyciflorus*	2	Mortality	1200	14
PCP (sodium salt)	*Brachionus calyciflorus*	2	Mortality	1200	15
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	430	17
PCP (sodium salt)	*Brachionus calyciflorus*	1	Mortality	1200	17
PCP (sodium salt)	*Brachionus rubens*	1	Mortality	620	16
PCP (sodium salt)	*Euchlanis dilatata*	1	Survivorship	60	28
PCP (sodium salt)	*Keratella cochlearis*	1	Survivorship	7.5	28
PCP (sodium salt)	*Lecane quadridentata*	1	Survivorship	10	28
PCP (sodium salt)	*Lepadella patella*	1	Survivorship	3.5	28
PCP (sodium salt)	*Philodina acuticornis*	1	Survivorship	300	28

Pesticide	Species scientific name	Test duration (days)	Effect	Concentration (µg/L)	Reference no.
PCP (sodium salt)	*Plationus patulus*	1	Survivorship	3.3	28
PCP (sodium salt)	*Trichocerca pusilla*	1	Survivorship	20	28
Vinclozolin	*Brachionus calyciflorus*	1	Mortality	30500	34

Table S 4. Chronic rotifer toxicity data for insecticides as compiled from the US-EPA ECOTOX database (http://cfpub.epa.gov/ecotox/).

Pesticide	Species scientific name	Test duration (days)	Endpoint	Effect	Concentration (µg/L)	Refererence no.
Fluazinam	*Brachionus calyciflorus*	2	LC10	Mortality	0.6	33
PCP_sodium salt	*Brachionus calyciflorus*	4	NOEC	Fecundity	10	35
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Progeny	110	32
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Progeny	190	32
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Progeny	330	32
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Progeny	450	32
PCP_sodium salt	*Brachionus calyciflorus*	4	NOEC	Fertilization	10	19
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Population growth rate	110	14
PCP_sodium salt	*Brachionus calyciflorus*	2	MATC	Population growth rate	140	14
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	110	15
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	30	36
PCP_sodium salt	*Brachionus calyciflorus*	2	MATC	Reproduction, general	80	36
PCP_sodium salt	*Brachionus calyciflorus*	2	NOEC	Reproduction, general	200	36
PCP_sodium salt	*Brachionus calyciflorus*	2	MATC	Reproduction, general	320	36

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Capítulo 2. Toxicity of abamectin and difenoconazole mixtures to a Neotropical cladoceran after simulated run-off and spray drift exposure

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Abstract

Aquatic risk assessments of pesticides in tropical countries have often been disputed for being largely based on risk evaluations conducted in temperate regions. Although pesticide sensitivity comparisons between temperate and tropical freshwater organisms have indeed not revealed consistent differences, it is currently still based on a relatively small tropical toxicity dataset. In addition, greater levels of runoff and spray drift may be expected in tropical agroecosystems, indicating that aquatic life in edge-of-field water bodies is likely to be subjected to higher concentrations of pesticides and their mixtures. The aim of the present study was to evaluate the toxicity of Kraft® 36 EC (a.i. abamectin), Score® 250 EC (a.i. difenoconazole) and their mixture to the Neotropical cladoceran *Macrothrix flabelligera*. Laboratory toxicity tests with the individual formulated products indicated EC_{50}-48h values of 3.1 and 659 µg a.i./L based as nominal test concentrations, respectively. Mixtures of the two pesticides revealed a dose-dependent deviation of the independent action model, with antagonism at low and synergism at high pesticide mixture concentrations. Laboratory toxicity tests were also conducted with microcosm water that was treated with the individual or mixtures through runoff or direct overspray. Microcosm tanks receiving runoff water from experimental soil plots applied with recommended doses of the individual pesticides did not show toxicity to the test organism. Microcosms that received runoff water containing the pesticide mixture, however, did cause a short-term effect on immobility. The microcosms that were treated by direct overspray of both the pesticide formulations also showed the most pronounced toxic effects. Study findings suggest a potential risk of these pesticides at environmentally relevant concentrations, especially when they are both present.

Keywords: Pesticide mixtures; microcosms; environmental risk assessment; *Macrothrix flabelligera*; tropics

1. Introduction

Pesticides applied to agricultural fields to increase their yield may contaminate adjacent watercourses via spray drift, run-off, drainage and/ or accidental spills (Capri and Trevisan, 1998). Developed countries, situated in temperate regions, are shifting towards reduced pesticide use as a result of improvements in agronomic practices, whereas developing countries, most of which are in tropical regions, are increasing their use of pesticides and fertilizers as they become wealthier (Sanchez-Bayo and Hyne, 2011; Lewis et al., 2016). Brazil, for example, became the world's top pesticide market consumer in 2008, accounting for approximately 20% of the total world use (Albuquerque et al., 2016). Despite this high use of pesticides in tropical countries like Brazil, there is still relatively little knowledge about the fate and toxicity of pesticides in tropical aquatic ecosystems as compared to temperate systems (Daam and Van den Brink, 2010; Sanchez-Bayo and Hyne, 2011; Carriquiriborde et al., 2014; Diepens et al., 2014; Lewis et al., 2016).

In the absence of data derived under (local) tropical conditions, risk assessments in tropical countries often rely on temperate toxicity data, although it may be debatable whether the fate and effects of chemicals are comparable in geographically distinct ecosystems (Daam and Van den Brink, 2010). Sensitivity comparisons of tropical and temperate species to pesticides have not demonstrated a consistent greater or lesser sensitivity of tropical species as compared to their temperate counterparts, although such comparisons are based on a relatively small tropical dataset (e.g. Maltby et al., 2005; Kwok et al., 2007; Rico et al., 2011). On the other hand, edge-of-field waterbodies in tropical agroecosystems have often been reported to be especially prone to pesticide contamination through runoff resulting from intensive irrigation practices and tropical rainfall (Daam and Van den Brink 2010; Lewis et al., 2016; Novelli et al., 2016). Furthermore, pesticides are often applied in close proximity to water bodies surrounding agricultural fields, resulting in relatively high levels of spray drift (Castillo et al., 1997; Daam and Van den Brink, 2010; Sanchez-Bayo and Hyne, 2011). Other frequently noted relatively high entry routes of pesticides in tropical countries are dangerous transportation and storage conditions, unnecessary applications and overuse, use of cheaper but more hazardous pesticides, and washing of application equipment in water bodies (Daam and Van den Brink, 2010 and references therein). Consequently, despite the absence of a clear difference in sensitivity, tropical freshwater organisms are likely to be subjected to higher (peak) pesticide concentrations and hence risks in real-world field conditions than their temperate counterparts.

The main Brazilian strawberry crop area in the municipality of Bom Repouso (Minas Gerais) has, a tropical climate by altitude, and can be classified as a monsoon-influenced humid subtropical climate according to Köppen´s classification. It is an agricultural area with intensive use of pesticides and previous field studies in this area identified the insecticide/acaricide Kraft® 36 EC (a.i. abamectin) and the fungicide Score® 250 EC (a.i. difenoconazole) as the main pesticides intensively used throughout the year (Nunes, 2010; Nunes and Espíndola, 2012). These pesticides are hence likely to occur simultaneously in edge-of-field water bodies in this region and this pesticide mixture may ensure greater toxic effects to aquatic life in these ecosystems as compared to their individual compounds.

The aim of the present study was to evaluate the toxicity of Kraft® 36 EC and Score® 250 EC to the Neotropical cladoceran *Macrothrix flabelligera*, a species native and of common occurrence in Brazilian freshwaters (Güntzel et al., 2003; Moreira et al., 2014). Laboratory toxicity tests were conducted with the individual compounds to establish their respective toxicity thresholds. Mixtures of both compounds were also tested to evaluate their combined effect and its underlying mechanism. The potential risks related with exposure to both compounds, alone and in combination, likely to occur in the field through runoff and spray drift was also evaluated through semi-field testing.

2. Materials and Methods

2.1. Test organism and culture conditions

Macrothrix flabelligera Smirnov, 1992 (Crustacea, Cladocera, Daphnidae) was initially isolated from the Lobo-Broa Reservoir (Itirapina, SP, Brazil) and had been kept in stock cultures for more than 4 years at the Ecotoxicology Laboratory of the Federal University of São Carlos (Brazil). The culture is maintained under controlled temperature (25 ± 1 °C) and photoperiod (12h light:12h dark; light intensity ± 1000 lux) in reconstituted water prepared according to standard ABNT (2005) with pH 7.0-7.8, hardness 40-48 mg $CaCO_3$/L, electrical conductivity ± 160 µS/cm. Although the ABNT (2005) protocol was originally developed for *Ceriodaphnia* spp, a previous study demonstrated that the same method may also be successfully used for *M. flabelligera* cultures (Moreira et al., 2014). Organisms were fed with 1 mL/L culture medium of an algal suspension of the microchlorophycean *Raphidocelis subcapitata* cultured in L.C. Oligo medium (10^5 cells/mL) supplemented with a mixture of fermented yeast and fish ration (Tetramin) at a ratio of 1:1 (Attachment, Figure 2,3 and 4)

(ABNT, 2005). Maintenance of cultures was performed three times a week by renewing the culture medium and subsequently adding the food mixture described above.

2.2. Runoff collection from contaminated soil plots

Four soil plots of 8 m^2 with a slope of 6% were set out 2 m apart from one another at the Center for Water Resources and Applied Ecology (CRHEA), located in the municipality of Itirapina, São Paulo state, Brazil (22º01'22"S, 43º57'38"W). Prior to the experiment, these plots were weeded and tilled. The plots contained loamy sand soil predominantly composed of fine sand (54%) and with an organic matter content of 13%.

Three of the plots were contaminated with the insecticide/acaricide Kraft® 36 EC (36 g abamectin/L; Cheminova, Brazil), the fungicide Score® 250 EC (250 g difenoconazole/L; Syngenta Crop Protection Ltda, Brazil), and a combination of both pesticides. A fourth plot was only sprayed with water to serve as a control (Attachment, Figure 5). To prevent cross-contamination, the control plot was covered with a plastic tarp during pesticide application to the treatment plots. Pesticide applications were made using a backpack sprayer, following the preparation instructions recommended for strawberry crop on the pesticide label: 30 mL Kraft® 36 EC 100/L pesticide application solution (0.1 L Kraft® 36 EC; 1.08 mg abamectin/m^2) and 0.40 mL Score® 250 EC 100/L pesticide application solution (0.02 L Score® 250 EC; 2 mg difenoconazole/m^2) (Mapa, 2016).

Immediately after the pesticide applications (< 1 h after application), a torrential rainfall event was simulated using water from the Lobo Reservoir next to CRHEA (pH: 7.02; conductivity: 16.1 µS/cm; suspended solids: 2.08 mg/L; turbidity 10 NTU; hardness: 6 mg $CaCO_3$/L and dissolved oxygen: 8.74 mg/L). The intensity (19 mm) was based on historical records from the weather station at CRHEA for the same period of the year as the experiment was conducted (summer; March-April 2015). The runoff water from a plot was collected in a 250-L polypropylene water tank dug in the ground at the lower end of the plot. Part of the runoff water was taken to the laboratory for physicochemical characterization and to conduct the toxicity tests with *M. flabelligera* and the rest of the runoff water was transferred to the outdoor microcosms at CHREA described in the next section.

2.3. Microcosm experiment

Each of the 26 microcosms consisted of a circular 250-L polypropylene tank (depth 0.58 m; diameter 1.2 m at the top and 0.95 m at the bottom), previously washed with subsequently 5% nitric acid, acetone and distilled water to avoid any influence from previous experiments. A 40-cm water column and ± 8-cm sediment layer in the microcosms originated from a mixture of three reservoirs: Vinte e Nove (São Carlos, SP), Lagoa Dourada (Itirapina, SP) and Broa (Itirapina, SP). Over an acclimatisation period of 6 months, a biocenosis was allowed to develop in the microcosms, although the toxicity tests performed in the present study refer only to *M. flabelligera*. Meanwhile, all microcosms were interconnected by tubes and the water was circulated using a pump with a flow rate of 4.6 L/min to achieve similarity between the communities in the systems.

To evaluate the impact of a single Kraft® 36 EC and Score® 250 EC contamination event (separately and as a mixture of both) through runoff obtained as described in subsection 2.2 and through direct overspray (Attachment, Figure 5), the following eight treatments were carried out:

1. Control (n=4) – **C**: no treatment;
2. Uncontaminated runoff control (n=4) – **UR**: treatment with runoff water from the uncontaminated soil plot;
3. Kraft-contaminated runoff – **RK** (n=3): treatment with runoff water from the Kraft-contaminated soil plot;
4. Score-contaminated runoff – **RS** (n=3): treatment with runoff water from the Score-contaminated soil plot;
5. Kraft and Score-contaminated runoff – **RKS** (n=3): treatment with runoff water from the Kraft and Score-contaminated soil plot;
6. Kraft spray drift – **SK** (n=3): Treatment by direct overspray of Kraft;
7. Score spray drift – **SS** (n=3): Treatment by direct overspray of Score;
8. Kraft and Score spray drift – **SKS** (n=3): Treatment by direct overspray of Kraft and Score.

Runoff treatments were made by replacing 25 L microcosm water (corresponding to approximately 10% of the water volume) with the corresponding runoff water. Spray drift treatments were made by direct overspray of the recommended doses of Kraft for strawberry crops (1.08 mg abamectin/m^2, corresponding to 83 mL Kraft) and/or Score (2 mg difenoconazole/m^2, corresponding to 16.6 mL Score). Direct overspray was evaluated since applications in the Brazilian strawberry crop area in the municipality of Bom Repouso (Minas

Gerais) are often made by airplanes. In addition, given the approximity of pesticide applications in tropical areas, spray drift has previously been set at 100% as a realistic worst-case exposure scenario in the tropics (e.g. Satapornvanit et al. 2004; Stadlinger et al. 2016).

Physical-chemical parameters were measured on a weekly basis. Temperature, pH, electrical conductivity and dissolved oxygen concentration were measured using a portable multiparameter probe model U-10 Brand Horiba (Japan). Turbidity was estimated by absorbance readings at a wavelength of 750 nm in a spectrophotometer (Hach, DR/2000) and suspended matter gravimetrically, after filtration of a known volume of microcosm water through glass fibre filters (GF/C - 47 mm diameter, 1.2 µm pore size; Teixeira et al., 1965). Total chlorophyll concentration was determined spectrophotometrically after extraction in hot ethanol (Nusch, 1980), whereas nutrients (total organic nitrogen and total phosphorus) were analyzed by spectrophotometry (APHA, 1995).

2.4. Laboratory toxicity tests

Acute 48h toxicity tests with *M. flabelligera* were conducted with Kraft 36 EC® and 250 Score EC® to establish their respective EC_{50} values, mixtures of these two commercial products, diluted and undiluted runoff water from the experimental soil plots, microcosm water throughout the experimental period and a reference substance (potassium dichromate). Besides an uncontaminated control (reconstituted water), test concentrations for the toxicity evaluation of Kraft 36 EC® (nominal 0.28, 0.56, 1.12, 2.25, 4.5 and 9 µg a.i./L) and Score 250 EC® (nominal 62.5, 125, 250, 500, 1000 and 2000 µg a.i./L) were set based on a series of preliminary tests for each compound. Mixture toxicity tests with these two commercial products were conducted with all 36 possible concentration combinations. Tests with the individual compounds and mixtures were conducted simultaneously to avoid any influence of eventual differences in sensitivity of test organisms used and experimental conditions. Acute toxicity tests with potassium dichromate were repeated 20 times, at intervals up to 30 days, whereas the tests with the individual commercial products were repeated five times. Tests with the pesticide mixtures, runoff water and microcosm water (on each sampling moment) were conducted once. In addition to these toxicity tests with the commercial products, toxicity of undiluted runoff water from the experimental soil plots as well as six serial concentrations (3.12%, 6.25%, 12.5%, 25%, 50%, 75%) were also evaluated. In addition, undiluted microcosm water filtered through a zooplankton net (mesh size: 68 µm) was tested throughout the experimental period:

one day before treatment, just after treatment on day 0 and 7, 14, 21 and 28 days post treatment. All tests started less than 10 hours after collecting the water samples.

To evaluate the physiological conditions of the test organisms, their sensitivity was tested by determining the EC_{50} of potassium dichromate ($K_2Cr_2O_7$). This reference substance is a compound routinely used in sensitivity tests (Environment Canada, 1990) although, to the best of our knowledge, there is no previously set sensitivity range with any reference substance for *M. flabelligera*. The reference test was repeated 20 times with test concentrations of 12.5, 25, 50, 100 and 200 µg potassium dichromate/L.

Test procedures followed the ABNT (2005) for *Ceriodaphnia* spp. (Crustacea, Cladocera). To this end, four replicates with 5 neonates (6-24 h old) each were placed in nontoxic polypropylene plastic cup containing 10 mL of test solution. Test duration was 48 hours and the experiment was conducted in an incubator (FANEM brand, São Paulo, Brazil) maintained at a constant temperature of 25 ± 1 °C in the dark. Animals were not fed during the experimental period. Water quality parameters (pH, temperature, electrical conductivity, hardness and dissolved oxygen) were measured at the start and at the end of the toxicity tests as described in the previous section. After 48 hours of exposure the organisms were observed under a stereomicroscope and the number of immobile individuals was counted and used to determine the EC_{50}-48h.

2.5. Chemical analysis of the test substances

To confirm nominal test concentrations, stock solutions were analyzed through high-performance liquid chromatography (HPLC Waters 2695), equipped with a diode array detector (DAD). The chromatographic analysis conditions were: Agilent Zorbax ODS C18 column (250 mm x 4.6 mm x 5 mm) and oven temperature of 25°C. The isocratic mobile phase utilized was methanol and water (0.1% formic acid; 95:5 v/v) for 6 min, at an injection volume of 20 mL and a flow rate of 1.0 mL min^{-1}. Analyses were carried out in three replicates. Based on absorbance signals observed in the DAD spectrum of the standard solutions, abamectin and difenoconazole were detected and quantified at 246 nm and 230 nm with retention times of 2.9 and 2.2 min, respectively. The precision in terms of repeatability, expressed as relative standard deviation (RSD), was 2.05% for abamectin and 1.88% for difenoconazole. Detection limits were 50 µg/L (abamectin) and 25 µg/L (difenoconazole).

2.6. Data analysis

For the acute toxicity tests conducted with runoff and microcosm water, normality (Shapiro-Wilk) and homogeneity 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 test Kruskall-Wallis, 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).

The EC_{50}-48h values for the acute toxicity tests conducted with the commercial products were calculated based on nominal test concentrations by nonlinear regression using the three-parameter logistic curve through the software Sigma Plot 11.0 (Systat, 2008) and Statistica version 7 (Statsoft, 2004). Data from the toxicity tests with the mixtures of these commercial products were analyzed through the conceptual models of concentration addition (CA) and independent action (IA). Initially, the observed data were compared with the combined expected effect calculated from the individual exposures using the MIXTOX tool (Jonker et al., 2005). The analysis was then extended, as described in Jonker et al. (2005) and the three deviations from the reference models synergetic/antagonistic interactions (S/A), deviation dose ratio-dependent (DR) and dose level-dependent (DL) were modelled by adding two parameters (“a” and “b”; Table 1). The parameter "a" becomes negative and positive in synergistic and antagonistic deviations, respectively. To describe the level dose level dependent (DL), another parameter (BDL) is included in addition to the parameter "a". In the latter case, the value of "a" indicates the deviation in high and low doses and the value of BDL indicates at what dose level the deviation changes. Further details on the deviation functions can be obtained from Jonker et al. (2005). The data were verified for these conceptual models and deviations, and the best fit was chosen through the maximum likelihood method. After identifying the statistically most appropriate model for the description of the deviation, the pattern of effects was deducted directly from the parameter values (Table 1) and the maximum deviation could be calculated in terms of effect level (Jonker et al., 2005; Freitas et al., 2014).

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	$b_i > 0$ - antagonism where the toxicity of the mixture is mainly caused by the toxic agent *i*
	a < 0 - synergism, except for those proportions of mixtures where a significant positive value of b indicates antagonism	$b_i < 0$ - synergy where the toxicity of the mixture is mainly caused by toxi*c i*
Dose level-dependent (DL)	a > 0 - low dose level antagonism and synergy in high dose level	$b_{DL} > 2$ - change in level of dose lower than EC50 $b_{DL} = 2$ - change in EC_{50}
	a < 0 - low dose level synergism and antagonism in high dose level	$1 < b_{DL} < 2$ - 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

3. Results and discussion

3.1. EC_{50} values for abamectin and difenoconazole

The mean EC_{50}-48h for *M. flabelligera* obtained from the twenty reference tests with potassium dichromate ($K_2Cr_2O_7$) was 54 ± 17 µg/L (mean ± SD) with a 95% confidence interval (CI) of 46-61 µg/L. All toxicity tests data met all the validity criteria laid down in the guidelines of the Brazilian Association of Technical Standards (ABNT, 2005). Physical-chemical conditions of the test solutions used were (minimum – maximum value): pH 7.0 - 7.7, water temperature 24.5 - 25.6 °C, electrical conductivity 152 - 159 µS/cm and water hardness 40 - 48 mg $CaCO_3$/L. At the end of all acute toxicity tests, immobility in controls was less than 10%.

In the acute toxicity tests evaluating the individual commercial products, EC_{50}-48h values for Kraft® 36 EC and Score® 250 EC were calculated separately for each of the five tests. Resulting EC_{50}-48h (mean ± SD) for Kraft® 36 EC averaged 3.1 ± 1.5 µg abamectin/L (95% CI 1.8 – 4.4 µg a.i./L) and for Score® 250 EC 659 ± 252 µg difenoconazole/L (95% CI 438 - 879 µg a.i./L) all based on nominal test concentrations.

3.2. Toxicity of mixtures of the commercial products

Abamectin is a mixture containing about 80% of avermectin B1a and 20% avermectin B1b, and these two components have similar biological and toxicological properties (Campbell, 1989). This insecticide/acaricide is known to act on the nervous system of arthropods, and has been indicated as a glutamate-gated chloride channel (GluCl) allosteric modulator (Casida and Durkin, 2013). The fungicide difenoconazole belongs to the chemical group of triazoles that inhibit the biosynthesis of sterol, a critical component for the integrity of cell membranes of fungi (Fishel, 2014; FRAC, 2016). Pesticide mixtures with similar mode of actions are generally considered to act through concentration addition, whereas the independent action (IA) model is used for mixtures with pesticides with different mode of actions (e.g. Jonker et al., 2005; Loureiro et al., 2010; Coors and Frische 2011; Altenburger et al., 2013; Silva et al., 2015). Subsequently, the IA model was applied in the present study to evaluate the response of *M. flabelligera* exposed to mixtures of Kraft® 36 EC and Score® 250 EC, and deviations from this model were evaluated using the methodology developed by Jonker et al. (2005) (Table 1).

As anticipated, the derived mixture toxicity data fitted the IA model, producing a sum of the squares of the residuals (SS) of 57.01 (p <0.05; $r2$ = 0.87; Table 2). After the addition of the parameters "a" and "b" to the IA model, a dose level dependent deviation of the IA model was noted to best describe the data, as can be deducted from the decrease in the SS value to 39.90 and the obtained correlation coefficient (p < 0.05; $r2$ = 0.91; Table 2). The results of the acute toxicity tests with Kraft® 36 EC and Score® 250 EC mixtures further show that antagonism occurred at low and synergism at high concentrations of these pesticide mixtures (Table 3; Figure 1). In addition, the results indicated that the change of the interaction occurred at concentrations lower than the EC_{50}-48h (Table 3; Figure 1). The toxic effects of mixtures include both toxicokinetic and toxicodynamic aspects and it is therefore necessary to understand the underlying species- and compound-related toxicity and clearance mechanisms, which may differ at different mixture concentrations (Loureiro et al., 2010).

Table 2. Summary of the analysis of the test of acute toxicity of mixtures of Kraft® 36 EC and Score® 250 EC for *Macrothrix flabelligera*.

	IA	S/A	DR	DL
max	0.83	0.80	0.80	0.81
β Kraft	2.55	3.17	3.28	1.77
β Score	2.54	3.45	3.27	1.68
EC_{50} to Kraft	2.57	3.51	3.77	2.91
EC_{50} to Score	591.75	803.69	742.04	682.07
a	-	-3.68	-1.73	8.93
$b_{DR/DL}$	-	-	-3.90	2.09
SS	57.01	51.33	50.33	39.90
r^2	0.87	0.88	0.88	0.91
χ^2 or test F	368.23	5.68	1.08	11.43
df	-	1.00	1.00	1.00
p (χ^2/ F)	2.03×10^{-78}	0.02	0.32	0.0007

max = maximum value of the response; β = slope the individual response dose curve; EC_{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.

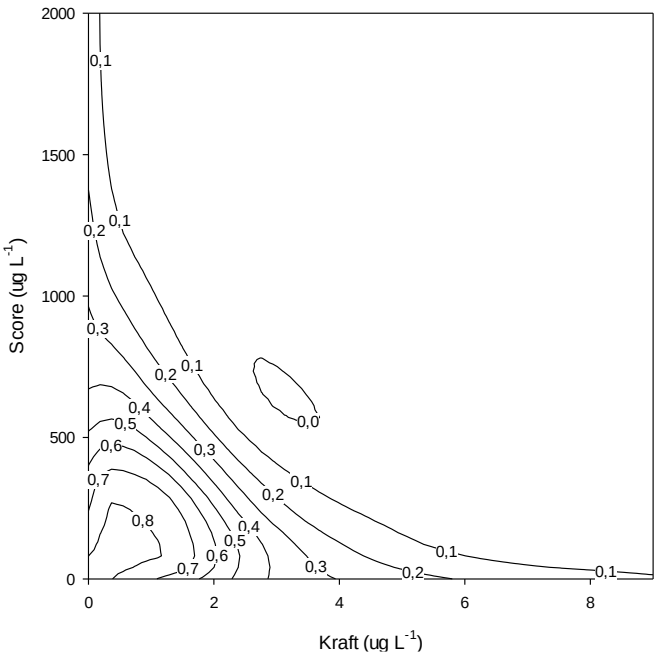

Figure 1. Isobologram of the effects of the pesticide mixtures on the mobility of Macrothrix flabelligera, demonstrating a dose level dependent (DL) deviation from the independent action (IA) that was analysed. The linear, concave and convex isoboles represent n no interaction, synergy and antagonism, respectively (Ryall and Tan, 2015).

In target fungi, difenoconazole is known to act by interfering with the ergosterol biosynthesis by inhibition of the C-14-demethylation of sterols, which leads to morphological and functional changes of the fungal cell membrane (EC, 2006b). Other sterol biosynthesis inhibitors like difenoconazole have been shown to impair the cytochrome P450 detoxification mechanism in insects (Pilling et al., 1995; Johnson et al., 2013). Few studies, however, have been conducted evaluating the toxicity of difenoconazole to other invertebrates (US-EPA, 2016) and the toxic mechanism in invertebrates hence remains largely unknown. For fish, it has been discussed that there is no indication that toxic effects of difenoconazole results from a specific mode of action other than general or systemic toxicity (EC, 2006b). In contrast to difenoconazole, the toxic action of abamectin and ivermectin, a synthetic derivative of abamectin, in invertebrates is well known to act through its effects on glutamate-gated chloride channels (EC, 2006a; Zemkova et al., 2014; IRAC, 2016). Zemkova et al. (2014) discussed that in most of the ivermectin-sensitive channels, the effects of ivermectin include the potentiation of agonist-induced currents at low concentrations and channel opening at higher concentrations.

Based on mutagenesis, electrophysiological recordings and functional analysis of chimeras between ivermectin-sensitive and ivermectin-insensitive receptors, these authors further concluded that ivermectin acts by insertion between transmembrane helices (Zemkova et al., 2014). Subsequently, these findings may possibly be related with the antagonistic and synergistic actions noted in the present study at low and high mixture concentrations, respectively (Table 3). The exact underlying toxic mechanisms, however, may only be elucidated through future studies at the molecular level.

Table 3. Immobility (in %) in the acute laboratory tests evaluating mixtures of Kraft 36 EC® (a.i. abamectin) and 250 Score EC® (a.i. difenoconazole).

Score® 250 EC (µg difenoconazole/L)	**Kraft® 36 EC (µg abamectin/L)**						
	0	0.28	0.56	1.12	2.25	4.5	9
0	7	13	20	33	40	60	87
62.5	13	40	20	20	53	93	93
125	20	13	13	20	33	87	100
250	53	27	7	40	67	80	100
500	47	20	40	67	80	100	100
1000	80	73	87	80	100	100	100
2000	93	87	100	100	100	100	100

Besides an interaction between the active ingredients, the formulation additives in the commercial products tested may evidently also have played a role. Commercial pesticides are generally a mixture of an active ingredient and a variety of other chemical compounds that aid in the mixing and dilution of the product and in its application and stability (Cox and Surgan 2006). The toxicity of formulated mixtures of pesticides may indeed be considerably influenced by formulation additives as evidenced in several studies (Coors et al., 2013; Chen and Stark, 2010). At present, however, limited knowledge is available concerning mixture effects of pesticide formulation additives, active substances and their transformation products (e.g. Altenburger et al., 2013; Coors et al., 2014).

3.3. Toxicity of runoff water from the experimental plots

In the acute toxicity tests where *M. flabelligera* was exposed directly to runoff water from the experimental soil plots, no toxic effects were observed in runoff water obtained from the control (RC) and Score® 250 EC-applied soil plots (RS) (Table 4). Statistically significant toxicity of runoff water from the pesticide-applied plots was only noted for the undiluted sample from the Kraft® 36 EC-applied plot and the two highest runoff concentrations from the plots receiving both pesticides (Table 4).

Table 4. Immobility (in %) noted in the laboratory tests with undiluted and diluted runoff water from the experimental soil plots. Runoff concentrations 100% and 0% refer to undiluted runoff water and control, respectively. Asterisks indicate significant differences from the control treatment at $p < 0.05$.

Dilution factor of the runoff tested (%)	Control	Score® 250 EC	Kraft® 36 EC	Kraft® 36 EC & Score® 250 EC
0	5	5	5	5
3	10	5	10	5
6	5	10	10	0
13	5	10	15	25
25	10	5	15	30
50	10	15	5	30
75	5	0	20	35*
100	10	5	80*	50*

The absence of toxicity in undiluted runoff control samples shows that runoff water and its constituents was not a stress factor to the organisms. A previous study at our facilities that evaluated the toxicity of Kraft® 36 EC (an older commercial product containing abamectin) to the daphnid *Daphnia similis* in runoff water from the same experimental plots as used in the present study also did not reveal toxicity of the control runoff water. Apparently, suspended

particles and dissolved compounds possibly present in the runoff water from the plots used (see Braun et al., 2012 for detailed characteristics) did not cause any physical or physiological effects leading to effects on mobility. In line with this, no toxicity in the runoff treatment was found by Novelli et al. (2012a) in a previous experiment conducted at our facilities assessing the toxicity of the insecticide/acaricide Vertimec® 18 EC (commercial formulation containing abamectin that was replaced by Kraft® 36 EC) with the daphnid *Daphnia similis*.

Abamectin and difenoconazole concentrations in the runoff water from the pesticide-applied soil plots could not be quantified since they were below their respective detection limits, i.e. 50 µg/L for abamectin and 25 µg/L for difenoconazole. At difenoconazole concentrations below this detection limit, no toxic effects would be anticipated based on the toxicity tests with single Score® 250 EC exposure (Table 3). This is hence in line with the absence of toxicity in the runoff water from the Score® 250 EC-applied plots (Table 4). The 80% immobility rate noted in the undiluted runoff water from the Kraft® 36 EC-applied soil plot (Table 4) would indicate that the abamectin concentration in this water was between 4.5 and 9 µg/L based on the results of toxicity tests with single Score® 250 EC exposure (Table 3). The toxicity tests with the pesticide mixtures further indicate that at abamectin concentrations between 4.5 and 9 µg/L, toxicity in the presence of a low difenoconazole concentration (93% immobilization at lowest concentration tested) is slightly greater than in its absence (60-87% immobilization). This may thus be related with the fact that diluted runoff water (concentration 75%) from the plots receiving both pesticides also showed toxicity (Table 4).

3.4. Microcosm water toxicity

Toxicity tests with microcosm water were performed in the laboratory one week before treatment and did not reveal toxicity to *M. flabelligera* in any treatment. The absence of toxicity in the microcosms treated with runoff from the control and Score® 250 EC-applied plots throughout the experimental period (Figure 2) is in line with the fact that the undiluted runoff water also did not reveal any toxicity to *M. flabelligera* (Table 4). Microcosms receiving 10% (v/v) runoff water from the Kraft® 36 EC-applied soil plot also did not show toxicity (Figure 2) and no effect at a 10% runoff dilution would indeed be expected based on the laboratory tests evaluating runoff dilutions from this plot (Table 4). Only microcosms receiving runoff water from the soil plot treated with both pesticides showed a significant effect on mobility a few hours after treatment (Figure 2). In the runoff dilutions toxicity evaluations, only the 75% and 100% runoff solutions of runoff water obtained from the plots treated with both pesticides had

shown significant toxicity (Table 4). However, although just not considered statistically significant ($0.06 < p > 0.1$), lower concentrations of runoff water obtained from this treatment including 13% (so close to the 10% runoff concentration in the microcosms) also revealed 25%-30% immobility (Table 4). This is hence in agreement with the 17% immobilized individuals of *M. flabelliger*a noted in the microcosm water receiving the mixture runoff treatment (Figure 2).

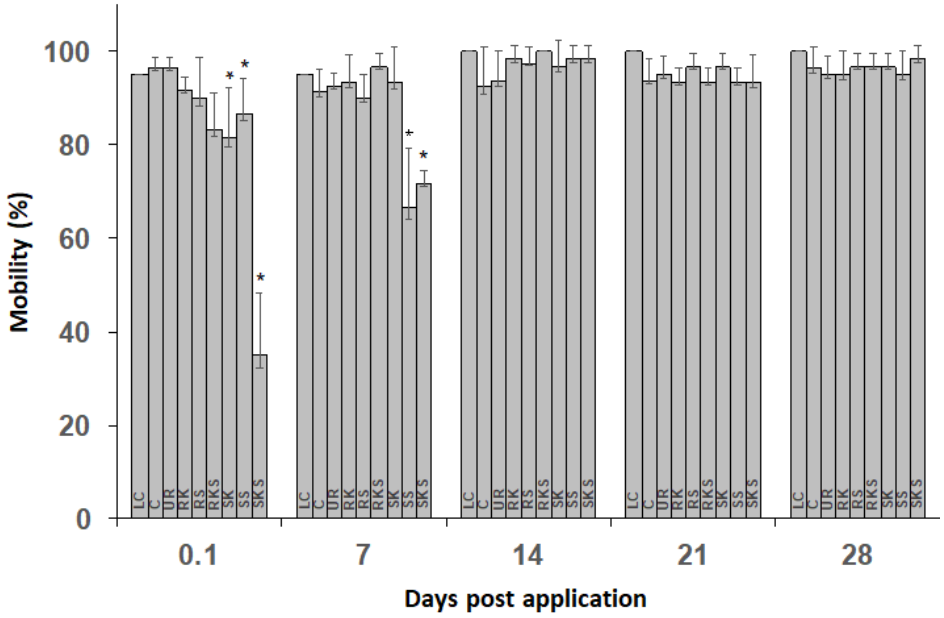

Figure 2. Mobility (mean ± SE) of the cladoceran *Macrothrix flabelligera* exposed to water from the microcosms receiving the following treatments: **LC** - Laboratory control; **C** - Control; **UR** – Uncontaminated runoff; **RK** - Runoff Kraft; **RS** - Runoff Score; **RKS** – Runoff Kraft + Score; **SK** - Spray drift Kraft; **SS** - Spray drift Score; **SKS** - Spray drift Kraft + Score.

Regarding the microcosms treated with the pesticides by direct overspray, simulating exposure through spray drift, nominal concentrations were 3.6 µg/L and 6.6 µg/L for abamectin and difenoconazole, respectively. Given the EC50 value of 3.1 µg/L established for abamectin, a greater immobility than the 18% noted in the KS treatment immediately after treatment was *a priori* envisaged at this nominal concentration (Figure 2). Furthermore, since abamectin has a high Koc value (5638 L/kg; EC, 2006a), it is likely that abamectin at least partly adsorbed to sediment or suspended particles and was subsequently not bioavailable to exert its toxicity.

Microcosms also sprayed with difenoconazole besides abamectin showed a greater toxicity (65% immobility) than when sprayed with abamectin alone, and toxicity also persisted until one week after application (28% immobility). This increase in toxicity of the mixture as compared to the single compounds was indeed also observed in the laboratory toxicity tests

evaluating the formulated products and run-off water (c.f. Tables 3 and 4 and text above). In line with the EC_{50} value obtained in the present study for difenoconazole (659 µg/L), no toxic effects were noted from microcosm water containing a nominal difenoconazole concentration of 6.6 µg/L a few hours after spraying them with only Score® 250 EC (SS treatment; Figure 2).

A week post application, however, a significant increased immobilization (33%) over controls was noted. As discussed above, the SKS treatment revealed a decrease in toxicity one-week post application, presumably resulting from dissipation processes (e.g. degradation and transformation). Subsequently, it is not likely that the toxicity observed in the SS treatment one-week post application was caused by difenoconazole, formulation additives of Score® 250 EC or eventual degradation products. No great differences in the various water quality conditions measured in the present study (c.f. the materials and methods section) were noted in microcosm water between the different treatments, with the exception of chlorophyll. The chlorophyll concentration in SS one-week post application was 686 µg/L, which was higher than the other treatments (except SKS: 690 µg/L) ranging levels of 176 to 314 µg/L. In addition, between the day of application and one-week post application, chlorophyll levels had increased by 50% in the SS treated microcosms. Since microcosm water samples were filtered through a zooplankton net (mesh size 68 µm), high chlorophyll levels in the SS microcosms samples taken one-week post application were also present in the laboratory test conducted with these samples. This may hence have caused a drop in oxygen through increased respiration because laboratory tests were conducted in the dark. Subsequently, this potential decrease in DO levels in the SS treatment may have exacerbated effects on mobility in the laboratory test.

3.5. Implications for risk assessment and concluding remarks

Difenoconazole has frequently been detected in edge-of-field waterbodies in Brazil and may be transported to surrounding water bodies like rivers (e.g. Montagner et al., 2014; Teló et al, 2015; Albuquerque et al., 2016). Due to its short half-life in water (4-6 h at the water surface and 4 days in the water column; Wislocki et al., 1989; Ali et al., 1997), abamectin is more difficult to detect in water bodies (Liu et al., 2011). However, its quick mode of action and high toxicity to aquatic organisms as demonstrated in the present and other studies (e.g. Casali-Pereira et al., 2015; Novelli et al., 2012b, 2016) indicate that management practices that reduce the potential risks associated with ecotoxicity of these highly toxic compounds need to be further developed. For example, integrated pest management to reduce repeated application, decrease the time of exposure of the non-target species and minimise the risk of pest resistance

can be introduced and programs to educate the public about these practices can be implemented (Bai and Ogbourne 2016).

The present study demonstrated that toxic effects on *M. flabelligera* are likely to occur at environmentally realistic exposure levels through simulated runoff and spray drift. True risk to aquatic life in the field is likely to be even greater than that noted in the present study since more sensitive aquatic organisms and higher exposure levels are likely to be present. For example, several cladoceran taxa have a greater sensitivity than *M. flabelligera* to abamectin, including the temperate *D. magna* (0.26-0.56 µg/L; EC, 2006a), the more widely distributed *D. similis* (0.0051 µg/L; Novelli et al., 2012b) and the Neotropical *Ceriodaphnia silvestrii* (1.47 µg/L; Casali-Pereira et al., 2015) (Figure 3A). For the fungicide difenoconazole, algae (*Scenedesmus subspicatus* and *Navicula pelliculosa*), crustaceans (*D. magna* and *Americamysis bahia*), and evidently fungi (*Alternaria sonali* and *Rhizoctonia solani*) are among the species that are more sensitive than *M. flabelligera* (Figure 3B).

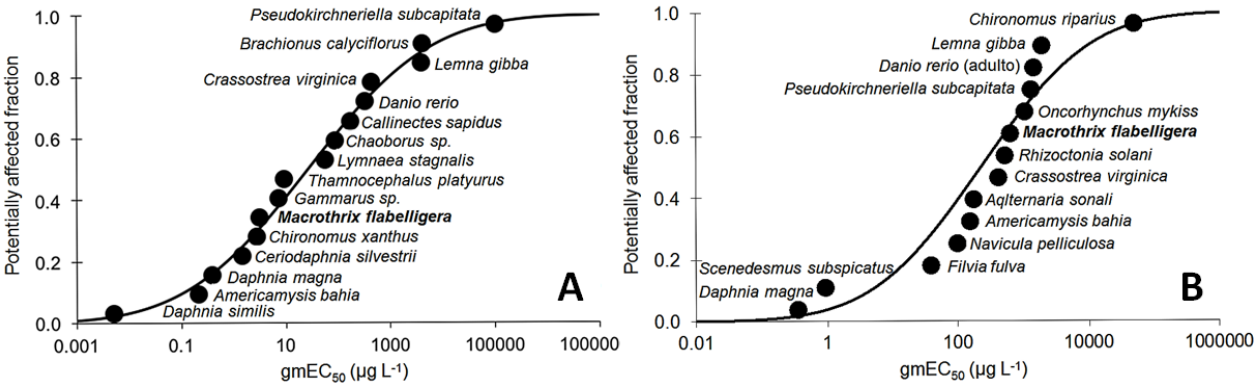

Figure 3. Species sensitivity distributions (SSD) constructed based on 48-EC_{50} values (geometric means) for abamectin (A) and difenoconazole (B) obtained in the present study for *Macrothrix flabelligera* (in bold), supplemented with data for other species from the US-EPA database (US-EPA, 2016). SSD curves were constructed as described in Vasconcelos et al. (2016).

In some cases, native tropical species have been demonstrated to be more sensitive than their temperate counterparts, e.g. for sodium and potassium by Freitas and Rocha (2011) and for pesticides (diazinon and methyl parathion) and metals (chromium and mercury) by Do Hong et al. (2004). Besides a possible different sensitivity, the use of indigenous species in tropical effect assessments has been recommended for several other reasons, including: i) a more ecologically relevant assessment of the true sensitivity and subsequently the potential risk of tropical freshwater life; ii) direct availability and hence less logistic constraints; and iii) to avoid introducing temperate exotics in tropical ecosystems (e.g. Daam and Van den Brink 2010;

Freitas and Rocha 2011; Rico and Van den Brink 2011). *M. flabelligera* is easily to obtain and maintain in the laboratory, toxicity test methods for this species are available and it has also previously been shown to be sensitive to other compounds (Mansano et al. 2016 and references therein). Subsequently, this species may be considered a good candidate to be included as a test species in tropical aquatic risk assessments where this species naturally occurs, such as Brazil.

The recommended doses for application in strawberry culture is to be repeated at intervals of 7 days for Kraft® 36 EC (Cheminova, 2016) and every 14 days to Score® 250 EC (Syngenta, 2016). In our study, only single applications of the recommended doses were considered in the spray drift and runoff simulations. Since overuse and bad agricultural practices are common in Brazilian crop areas (e.g. Nunes, 2010), the actual risk under actual exposure profiles in the field is likely to be greater. More research is needed into pesticide fate in tropical agroecosystems to assess real-world pesticide exposure and to evaluate to what extent pesticide fate models and scenarios developed for temperate regions (e.g. FOCUS, 2001) can be used or adapted for use in tropical settings (Daam and Van den Brink, 2010; Sanchez-Bayo and Hyne, 2011; Lewis et al., 2016). Due to the fast degradation of abamectin and logistic constraints discussed above, pesticide exposure could not be established throughout the course of the experiments conducted in the present study. Efforts need hence to be made in future studies to implement chemical analytical techniques that enable characterizing the pesticide exposure profiles in the field and subsequently comparing these with results from pesticide fate model simulations.

Studies in Brazil and elsewhere have reported the simultaneous use of formulated products containing difenoconazole and abamectin in agricultural areas near adjacent water bodies (e.g. Milhome et al., 2009; Nunes, 2010; Thuy et al., 2012). This study demonstrated that mixtures of these pesticides caused greater toxicity (synergism) to the cladoceran *M. flabelligera* than when tested individually. Prospective environmental risk assessments and environmental quality criteria based on individual exposures may hence not adequately protect aquatic ecosystems. Although there has been a great increase in research on mixture toxicity over the past few years, additional information is required to develop practical criteria for selecting pesticide mixtures that require additional (Legislative) attention based on their likelihood to exert synergistic responses atconcentrations likely to occur in the field (e.g. Altenburger et al., 2013; Coors et al., 2013, 2014; Silva et al., 2015).

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Capítulo 3: Effects of abamectin and difenoconazole and their mixtures on *Daphnia magna*: a multiple endpoint approach (biochemical biomarkers, energy reserves and reproduction)

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Abstract

The aim of this study was to evaluate the toxicity of the Kraft® 36 EC (a.i. abamectin) and the Score® 250 EC (a.i. difenoconazole) and their mixtures on *D. magna* at different biological levels. The survival (EC_{50}), reproduction and biochemical biomarkers**:** cholinesterase (ChE), catalase (CAT) and lipid peroxidation (LPO) were evaluated. Energy-related parameters (total proteins and lipids) were also studied together with energy consumption (EC). To achieve this goal daphnid neonates were exposed for 96-h to three concentrations of Kraft® 36 EC (2, 4 and 6 ng a.i./L) and Score® 250 EC (12.5, 25 and 50 µg a.i./L) for the biochemical experiments and during 15 days to five concentrations (1, 2, 3, 4 and 5 ng a.i./L) of the insecticide and (3.12, 6.25, 12.5, 25 and 50 µg a.i./L) of the fungicide. A full factorial design has been selected that included a test for each individual compound and a set of combinations of both for the mixture experiments. Our data have shown effects on different parameters when *D. magna* is exposed to low concentrations of these pesticides, especially in mixture. Isolated concentrations of compounds did not cause effects to antioxidant and detoxifying enzymes, except for LPO that was induced in the largest concentration of difenoconazole tested. For ChE and CAT there was induction in mixture treatments, occurring in minor concentrations for CAT and on the two largest for ChE. With regard to energy reserves, there were no significant differences between treatments for total proteins but there were for lipids, in highest concentrations of mixture combinations, whereas for EC, all treatments were affected. Although increases in reproduction occurred for isolated difenoconazole treatments and mixtures, only for one treatment of mixture it was significant, producing 25% more newborns per female than in the control. Findings from this study suggest a potential risk of the pesticides evaluated at environmentally relevant concentrations, especially when they are both present.

Keywords: Kraft® 36 EC, Score® 250 EC, Pesticide mixtures, Cholinesterase, Oxidative stress.

5. Introduction

The impact assessment of a stressor on aquatic ecosystems requires the evaluation of potential effects at different levels of biological organization, from the molecular level to the population and community levels (Moore et al., 2004; Rodrigues et al., 2017). At the molecular level, several biochemical biomarkers have been used as efficient tools for toxicity monitoring due to their sensitivity, speed and accuracy (Morgan et al., 1999). They can be used as initial indicators of environmental pollution and consequent effects since they can be detected at very low concentrations in the bodies of test organisms (Coelho et al., 2011). Metabolic costs resulting from xenobiotics-induced stress may, especially under long-term exposures, induce depletion of energy reserves and subsequently adversely affect growth or reproduction of individuals and ultimately damaging the population structure and dynamics (de Coen and Janssen, 2003). These metabolic costs and depletion of energy reserves may also be determined through molecular biomarkers.

Over the past decades, the use of pesticides has been considered as one of the main pollution sources of aquatic ecosystems worldwide (Shinn et al., 2015; Silva et al., 2015). A fact that may exacerbate the risks of water body contamination is that in many cases pesticides are applied as mixtures in agricultural crops to increase the effectiveness of the pest control (Junghans et al., 2006). However, mixtures of these compounds may cause additive or synergic effects on non-target aquatic communities (Axelrad et al., 2002; Coors and Frische, 2011; Deneer, 2000; Gatidou et al., 2015; Verro et al., 2009). Furthermore, the risk assessment of these complex mixtures can be underestimated because risk assessment procedures generally only evaluate the toxicity of each chemical product individually and generally do not account for their additivity or possible interactions (Laurel et al., 2010; Pavlaki et al., 2011).

The main Brazilian strawberry crop area in the municipality of Bom Repouso (Minas Gerais) is an agricultural area with intensive use of pesticides and previous field studies in this area identified the insecticide/acaricide Kraft® 36 EC (a.i. abamectin) and the fungicide Score® 250 EC (a.i. difenoconazole) as the main pesticides used (Nunes, 2010; Nunes and Espindola, 2012). Abamectin is an avermectin that operates in the receptors of gamma-aminobutyric acid (GABA) in both invertebrates and vertebrates (Novelli et al., 2016; EC, 2006a.). Difenoconazole is an azole fungicide known to act by interfering with the biosynthesis of ergosterol in target fungi, leading to morphological and functional alterations of the fungal cellular membrane (Campbell, 1989; EC, 2006b). Since both pesticides are intensively used

throughout the year, they are likely to occur simultaneously in edge-of-field water bodies in this region.

The difenoconazole was frequently detected in shallow water near agricultural areas around the world, and may be transported to the surrounding water bodies, as rivers (Montagner et al., 2014; Teló et al., 2015; Albuquerque et al., 2016). For example, in irrigated areas of rice crops in Brazil (Rio Grande do Sul), difenoconazole residues were detected up to 20 days after the application of the product, with concentrations varying from 400 to 36200 µg/L (Teló et al, 2015). In Malaysia (Kedah), values of 300 µg/L (Latiff et al., 2010) were recorded. In China (Changsha, Changchun, Hangzhou), reported concentrations from 1980 to 2910 µg/L (Zhang et al., 2011). In surface waters of rivers and streams in Australia (Victoria), Schäfer et al. (2011) registered the value of 0.15 µg/L. Satapornvanit et al. (2004), when assessing the risks of the use of pesticides in aquatic ecosystems adjacent to monoculture of fruits growing in Thailand (Nong Sua), concluded that the *predicted* environmental concentration (PEC) in this region is 28 µg/L for difenoconazole. With respect to the abamectin, it is more difficult to detect it in water bodies (Liu et al., 2011) due to its short half-life in water (4-6 hours on the water surface and 4 days in the water column (Wislocki et al., 1989; Ali et al., 1997). However, its rapid mode of action and high toxicity for aquatic organisms, as demonstrated in several studies (e.g., Casali-Pereira et al., 2015; Novelli et al., 2012, 2016; Moreira et al., 2017; Sanches et al., 2017), indicate that management practices to reduce the potential risks associated with the ecotoxicity of these highly toxic compounds are yet to be developed. Since both pesticides are used intensively throughout the year, they are likely to occur simultaneously in water bodies near monocultures and this mixture of pesticides may have greater toxic effects for aquatic life in ecosystems than the individual compounds.

Mixtures containing abamectin and difenoconazole may have greater toxic effects to aquatic organisms than their individual compounds. Moreira et al. (2017), for example, noted synergistic effects of high binary mixture concentrations containing abamectin and difenoconazole on the mobility of the Neotropical cladoceran *Macrothrix flabelligera*. Mixtures of these compounds also showed a synergistic interaction when their effects on the survival of the zebrafish (*Danio rerio)* were evaluated (Sanches et al., 2017). To the best of our knowledge, however, the effects of this pesticide mixture has never been assessed on a molecular level. Subsequently, the mechanistic pathway of this synergistic effect based on uptake and elimination of the toxicant (toxicokinetics) and damage and repair processes (toxico dynamics) within the organism remains unknown.

Within this context, the objective of the present study was to evaluate the toxicity of abamectin and difenoconazole pesticides isolately and in mixtures on *Daphnia magna* from the biochemical and energetic aspects and at different biological levels, from individual to population levels. In acute toxicity tests, the mobility (EC_{50}) was evaluated whereas in chronic toxicity tests the endpoints evaluated were reproduction and selected biochemical biomarkers related with neurotransmission activities, cholinergic excitation (cholinesterase-ChE) and oxidative stress (catalase of antioxidant enzymes (CAT) and lipid peroxidation (LPO)). Energy-related parameters (proteins and total lipids) were also studied together with energy consumption via electron transport system.

6. Materials and methods

6.2. Test organism and culture conditions

Daphnia magna were obtained from in-house cultures that have been kept in the laboratory of Applied Ecology and Ecotoxicology of the University of Aveiro, Portugal for several generations. Organisms were maintained in synthetic medium of hard water ASTM (ASTM, 1980; USEPA, 2002), designated from now on as ASTM medium, supplemented with an organic additive compound made up with *Ascophyllum nodosum*, seaweed extract and vitamins (Baird et al., 1989).

Cultures were maintained under a controlled temperature of 20 ± 2 °C, a photoperiod of 16 h light/8 h dark. The ASTM medium was renewed and the organisms were fed three times a week, at the concentration of 3.0 x 10^5 cells mL^{-1} of the chlorophycean microalga *Raphidocelis subcapitata*, which was cultivated in MBL medium in the same laboratory controlled conditions (Stein, 1973). Neonates (≤ 24 h) from the third to fifth brood of a a healthy progenitor stock were used as test-organisms.

6.3. Chemicals and test concentrations

The commercial formulation Kraft® 36 CE (36 g abamectin/L; Cheminova, Brazil) and the fungicide Score® 250 EC (250 g difenoconazole/L; Syngenta Crop Protection Ltda, Brazil) were used. The stock solutions of commercial abamectin (1 mg a.i./L) and difenoconazole (100 mg a.i./L) were made by diluting a specific quantity of each compound in ultrapure water (Milli-Q) immediately before the start of the tests. The same concentrations of the stock solution were

used in all toxicity tests. Nominal concentrations of each test compound were obtained by dilution of the stock solution in ASTM culture medium.

6.4. Acute and chronic toxicity tests

Acute toxicity tests were conducted according to established standard protocols (ASTM, 1997; ISO, 1996; OECD 202, 2000). Based on the results of preliminary range finding tests, the following nominal test concentrations for each compound were selected: 0, 12, 24, 48, 96, 192 and 384 ng abamectin/L administered as Kraft® 36 EC and 0, 50, 100, 200, 400, 800 and 1600 μg difenoconazole/L administered as Score® 250 EC. Four replicas were used for each of the five pesticide treatments and the uncontaminated control. Each replica consisted of a glass vial (50 mL) containing five neonates (6-24 h age) and 50 mL test solution or reconstituted ASTM water (control).

The experiments were conducted at 20 ± 2 °C in darkness and animals were not fed during the test. After the 48 h exposure period, the number of immobilized and/or dead individuals were counted and used to determine the median effective concentration (EC_{50} 48-h). Individuals that were unable to swim within 10 s after gentle agitation of the test container were considered immobilized. Three tests were conducted for each compound.

The chronic toxicity tests were conducted according to standard protocols (ASTM, 1997; ISO, 2000; OECD 2111, 2012) except that the exposure time was modified to 15 days (until 3rd brood) based on the study of Ribeiro et al. (2011) and Vacchi et al. (2016). The following nominal concentrations were tested: 0, 1, 2, 3, 4 and 5 ng abamectin/L administered as Kraft® 36 CE and 0, 3.12, 6.25, 12.5, 25 and 50 μg difenoconazole/L administered as Score® 250 EC. The chronic toxicity test was conducted using four replicas, each containing one neonate (6-24 h) in 50 mL test solution or 50 mL reconstituted water (control). The organisms were fed and maintained under the same conditions (temperature and photoperiod) as described above for culture maintenance. The solutions were renewed every other day after which the number of surviving adults and neonates was recorded.

6.5. Exposure tests for the realization of biochemical tests and energy-related parameters

Methods for short-term tests (96 h) evaluating the effects of the insecticide and the fungicide on biochemical and energy-related parameters were conducted through modificated standard protocols (ASTM, 1997; ISO, 1996; OECD 202, 2004), under the same temperature

and photoperiod as described for cultivation. *D. magna* neonates (6-24 h) from a stock culture were exposed to three concentrations of each compound, i.e. 0, 2, 4 and 6 ng abamectin/L administered as Kraft® 36 EC and 0, 12.5, 25 and 50 µg difenoconazole/L administered as Score® 250 EC. These test concentrations were set since they were noted as subletal (EC_{10}-48 h) in the acute trials with these compounds (see section 2.3), as confirmed with literature data and preliminary tests.

The daphnids were exposed in glass beakers of 1000 mL containing 800 mL test solution. Organisms from the stock culture were randomly distributed to each treatment beaker, which contained 75 organisms for exposure to isolated compounds and 45 organisms for the exposures to the mixtures. During the exposure period, the animals were fed, with food renewal every 48 hours.

At the end of the exposure, the organisms were transferred to eppendorf tubes in groups of 15 organisms per eppendorf, totaling five replicates for the isolated tests and three replicates for the mixtures, for both, biomarkers and the energy reserves. Immediately after transferring the organisms to the eppendorfs, they were frozen in liquid nitrogen and stored at -80 °C until homogenization for the performance of enzyme determinations.

6.6. Mixture Toxicity tests

For chronic mixtures toxicity tests and for short-term exposure tests for subsequent completion of the biochemical tests and energy reserves of the mixture of abamectin and difenoconazole a complete factorial design was selected that included a test for each individual compound and a set of combinations of both being 25 combinations for the chronic toxicity test and a set of 9 combinations for biochemical and energy-related tests (Figure 1). Tests with individual compounds were simultaneously carried out with mixtures, so that differences in the responses of organisms due to sensitivity variations could be controlled and did not invalidate analysis.

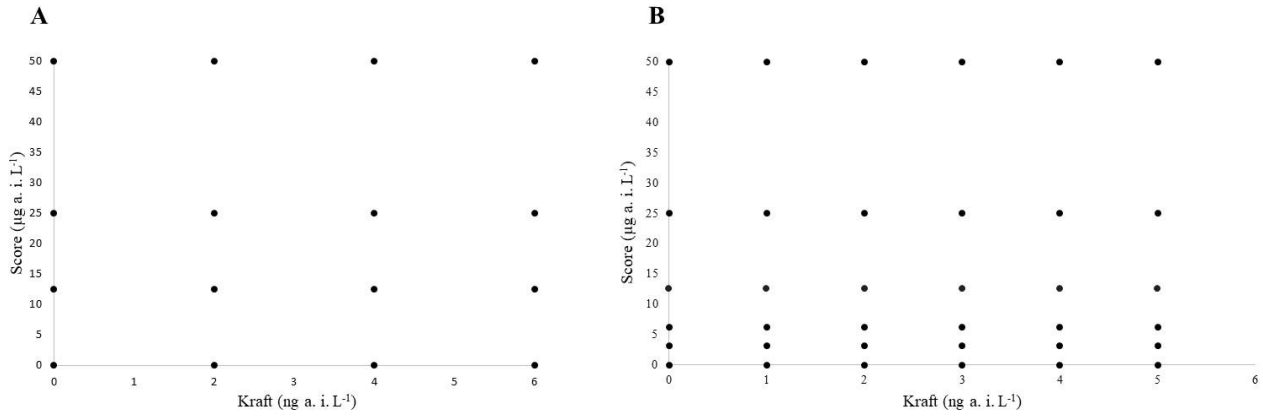

Figure 1. Factorial design adopted to select the test concentrations in the single and mixture toxicity tests with Kraft® 36 EC (a.i. abamectin) and Score® 250 EC (a.i. difenoconazole): A) Biochemical assays and B) Chronic toxicity test.

6.7. Biochemical assay and energy reserves

The total soluble ChE activity was determined according to Ellman et al. (1961), adapted to the microplate, as described and optimized for *D. magna* in Guilhermino et al. (1996). Samples for determining the activity of ChE (15 organisms in 300 µL of buffer) were homogenized in phosphate buffer (0.1 M, pH 7.2) using a rotary tissue homogenizer at 14,000 rpm, maintained in ice during homogenization. The homogenates were subsequently centrifuged at 6000 rpm for 3 min at 4 °C and then diluted in the proportion 1:5 after which the enzymatic activities were measured by means of a spectrophotometer Thermo Scientific Multiskan EX (Ascent Software 2.6) at 412 nm. The enzyme activity was expressed as nmol of the complex formed per minute per milligram of protein.

Catalase (CAT) was spectrophotometrically determined by measuring the decrease in absorbance at 240 nm (ε 240 = 0.00394 ± 0.0002 mm^{-1} mm^{-1}), as described by Aebi (1984) due to the decomposition of H_2O_2 for H_2O and O_2. To this end, fifteen organisms were homogenized in 300 µL phosphate buffer and then samples were diluted at a 1:50 proportion in buffer pH 7.0. Subsequently, the absorbance of the sample was monitored spectrophotometrically at 240 nm during 30 s, and the activities were expressed in nmoles of H_2O_2 consumed per minute, per milligram of protein.

Lipid peroxidation (LPO) was determined spectrophotometrically by measuring the decrease in absorbance at 535 nm (ε = 1.56 $x10^5$ $M^{-1}cm^{-1}$) as described by Bird and Draper (1984) and Ohkawa et al. (1979). Initially, 15 organisms were homogenized in 300 µL phosphate buffer. LPO were expressed by the concentrations of TBARS which were derived

from an outer standard curve of 1, 1, 3, 3-Tetrametoxipropano and the values expressed as nanomols of TBARS formed per mg of wet weight.

The protein concentration of the specimens was determined according to the spectrophotometric method (wavelength 595 nm) of Bradford (1976), adapted to the microplate using the Y-globulin as a standard, in order to enable expressing the enzymatic activities on the basis of protein content. To determine total protein content, lipid content and Ec (energy consumed), the protocol described in de Coen and Janssen (1997) with adaptations was used. Initially, 15 organisms were homogenized in 1 mL of Milli-Q water using a rotary tissue homogenizer at 14,000 rpm. For total protein content measurements, the homogenate was mixed with trichloroacetic acid 15% (TCA) after which samples were incubated at -20ºC for 10 min. Then samples were centrifuged (3500 rpm, 10 min, 4ºC). The pellet was re-suspended in 625 µL sodium hydroxide (NaOH), incubated at 60ºC for 30 min, after which it was neutralized with 375 µL hydrochloric acid (HCl), and then used for measuring the protein fraction. Total protein was determined using Bradford's reagent and the absorbance was measured at 590 nm in a microplate reader, using bovine serum albumin as a standard. The protein content was expressed as mJ/org.

Total lipids were determined following an adapted protocol of that described in Ferreira et al. (2015). Here 500 µL of chloroform were added to the 300 µL of fraction and vortexed, after which 500 µL of methanol and 250 µL ultra-pure water were added and the samples centrifuged (3500 rpm, 5 min, 4ºC). The top phase was discarded and the bottom phase containing the lipid extraction was used for lipid content measurements. To this end, the previous phase was diluted in 500 µL of H_2SO_4 and heated for 15 min at 200ºC. After cooling down, 1.5 mL ultra-pure water was added. The absorbance was measured at 375 nm and lipid content was expressed as mJ/org.

For the electron transport activity, 150 µL of a buffer containing 0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vynil Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100 were added to 300 µL homogenate. Samples were centrifuged (3500 rpm, 5 min, 4ºC) and the supernatant was used as sample. In a microplate, to each 50 µL of the sample, 150 µL buffered substrate solution (0.13 M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH) was added. The reaction started by adding 100 µL INT (p-IodoNitroTetrazolium, 8 mM). The absorbance was measured at 490 nm for 3 min. The amount of formazan formed was calculated using a molar extinction coefficient of 15,900 M^{-1} cm^{-1}. The values of Ec were calculated according to Verslycke et al. (2004), i.e. Ec = activity of the Electron Transfer System (ETS) (mJ/mg org./h).

6.8. Data analysis

The values of EC_{50}-48 h, EC_{10}-48 h and EC_{20}-48 h with their 95% confidence intervals for the acute toxicity tests were calculated with the three-parameter logistic curves using the SigmaStat software (Systat, 2008). The statistical analysis of the fertility parameter (number of neonates per female) in the chronic toxicity test and the biochemical parameters were carried out through the analysis of variance (ANOVA) using the R (the R Foundation for statistical Computing, http://www.R-project.org) software. Multiple post-hoc comparisons (Tukey test) were performed for data presenting normal distribution and homogeneity of variances. In all statistical tests, a significance level of 95% ($p \leq 0.05$) was adopted. The confirmation of the homocedasticity and normality of the errors were checked through graphs of Half-normal probabilities with envelope simulations (Demétrio and Hinde, 1997; Hinde and Demétrio, 1998) through the HNP package of the software R (Moral et al, 2017). LPO data were square-root transformed to correct for normality.

7. Results and discussion

7.1. EC_{50}, EC_{20} e EC_{10} values for abamectin and difenoconazole

In the acute toxicity tests evaluating the individual compounds, the values of EC_{50}, EC_{20} and EC_{10}-48 h for immobility of *D. magna* were calculated separately for each of the three replicated tests performed. The averaged values and their respective 95% confidence intervals are presented in Table 1. All data from the toxicity tests fulfilled the validity criteria laid down in the guidelines followed (ASTM, 1997; ISO, 1996; OECD 202, 2000). The physical and chemical conditions of the test solutions used were (minimum-maximum value): pH 7.3-7.5 and water temperature 21.0-22.0 °C. At the end of all acute toxicity tests, the immobility in controls was less than 10%.

Table 1. EC_{50}, EC_{20} and EC_{10}-48 h mean values for Kraft®36 EC and Score®250 EC (± SD) with a 95% confidence interval (CI). All based on nominal test concentrations.

	EC_{50}-48 h	**EC_{20}-48 h**	**EC_{10}-48 h**
Kraft® 36 EC (ng a.i./L)	119,01 (± 1,1) 75,4 - 158,4	41,22 (± 1,2) 12,9 - 67,6	22,1 (± 1,6) 1,7 - 44,3
Score® 250 EC (µg a.i./L)	580,04 (± 21,5) 354,7 - 787,5	218,8 (± 32,05) 62,6 - 395,6	124,1 (± 26,7) 7,61 - 270,6

The species sensitivity distribution (SSDs) constructed by Moreira et al. (2017) based on EC_{50}-48 h values for abamectin and difenoconazole previously demonstrated the high sensitivity of *D. magna* to these compounds as compared to other aquatic organisms. These SSDs show that *D. magna* was the third most sensitive species to abamectin and even the most sensitive species to difenoconazole from all species included in the SSD analysis (Moreira et al., 2017).

Abamectin and ivermectin, a synthetic derivative of abamectin, are known to act through the channels of glutamate chloride (EC, 2006a, Zemkova et al., 2014, IRAC, 2017) in both vertebrates and invertebrates. Zemkova et al. (2014) argued that in most ivermectin-sensitive channels, the effects of ivermectin include potentiation of agonist-induced currents at low concentrations and channel opening at higher concentrations. In target fungi, difenoconazole is known to act through interaction with ergosterol biosynthesis by inhibiting the demethylation of C-14 sterols, leading to morphological and functional alterations of the fungal cell membrane (EC, 2006b). Some azoles fungicides impair the detoxification mechanism of cytochrome P450 in insects (Pilling et al., 1995; Johnson et al., 2013). Given their insecticidal and fungicidal modes of action, the lower value of EC_{50} for abamectin as compared to difenoconazole was indeed anticipated a priori (e.g., Maltby et al., 2005; Table 1).

7.2. Chronic toxicity test: reproduction

The number of newborns in *D. magna* (15 days old) differed only for one mixture treatment (M14) ($F = 3{,}723$, $p < 0.05$), in which it represented 25% more newborns when compared to the control (Figure 2). No interaction was found between the two compounds for the other mixture combinations nor were effect found when the compounds were tested individually. Although for some treatments the number of neonates was greater than in the controls, these differences were not significantly significant.

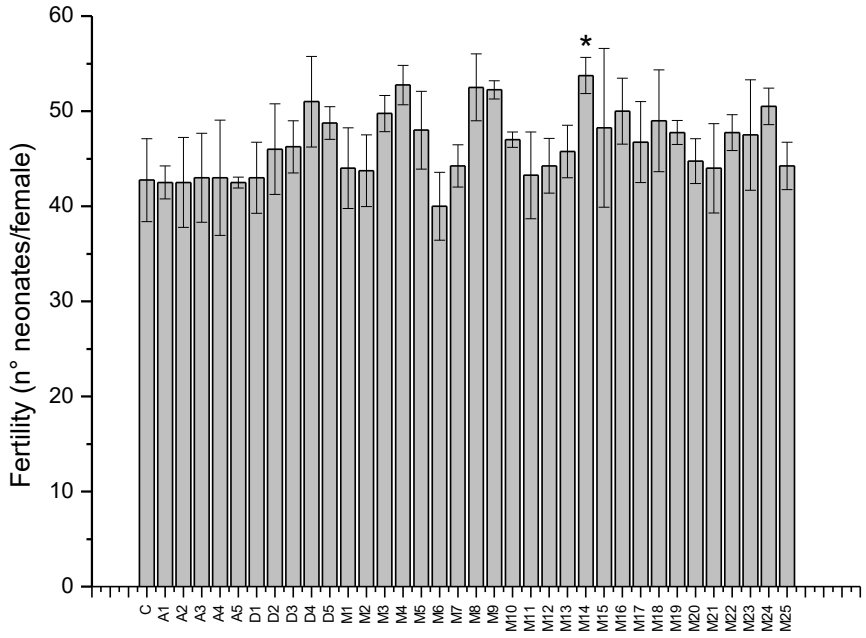

Figure 2. Fertility of *Daphnia magna* (mean ± SD number of neonates per female) after exposure 15 days to different concentrations of abamectin dosed as Kraft® 36 EC (A 1-5), difenoconazole dosed as Score® 250 EC (D 6-10) and their mixtures (M11-35) in chronic toxicity tests. The asterisk indicates the value significantly different from control ($p \leq 0.05$, Tuckey's test).

Several other studies have reported effects of low pesticide concentrations on the reproduction of cladocerans, either by reducing the number of newborns or by increasing embryo abnormalities (eg. Palma et al., 2009; Freitas and Rocha 2012). For example, for one of the studied compounds, abamectin (Casali-Pereira et al., 2015) and for the herbicide, the diuron (Mansano et al., 2016), both in *Ceriodaphnia silvestrii*.

The stimulating response at low concentrations to fertility in the called hormesis process is induced directly or as a result of compensatory biological processes by the initial interruption of homeostasis (Calabrese and Baldwin 2002). Hormesis has been shown in several invertebrates exposed to sublethal pesticide concentrations (for example, Zalizniak and Nugegoda 2006; Cedergreen et al., 2007; Li and Tan, 2011; Moreira et al.2015). Studies have suggested different mechanisms to explain hormesis, which are usually related to increased acquisition or changes in energy allocation (eg., Forbes 2000, Jager et al., 2013, Tyne et al., 2015). Although the increased fertility of *D. magna* after exposure to the fungicide and some of its combinations with the insecticide can be interpreted as a positive response, this could have serious consequences for population survival and fitness through a combination of disproportionate energy allocation to reproduction and stress caused by such exposure.

7.3. Biochemical Biomarkers

We measured the activities of biomarkers of neurotransmission cholinergic excitation (cholinesterase-ChE) and related to oxidative stress, catalase of antioxidant enzymes (CAT) and lipid peroxidation (LPO) in individuals of *D. magna* after 96h of exposure. The concentrations of Kraft ® 36 CE and Score® 250 EC are specified in Figure 1.

For ChE's activity, significant differences were encountered in two of mixture treatments (M8 and M9) ($F = 6{,}857$, $p < 0.05$) (Figure 3A). For CAT, significant differences were observed for the treatments of mixtures M1 and M3 ($F = 4.7$, $p < 0.05$) (Figure 3B). With regard to LPO, the D3 treatment presented significant difference (Figure 3C) ($F = 24.89$, $p < 0.05$), being the largest concentration tested of difenoconazole (50 µg/L).

While the lower concentrations tested separately from the compounds did not cause significant modulation of antioxidant effects and enzymatic detoxifying, the mixtures caused the effect at low concentrations when analyzed the ChE and CAT. In all cases, exposure to compounds induced a significant increase of 5 times of ChE, 2 times CAT and LPO (this one only for the fungicide).

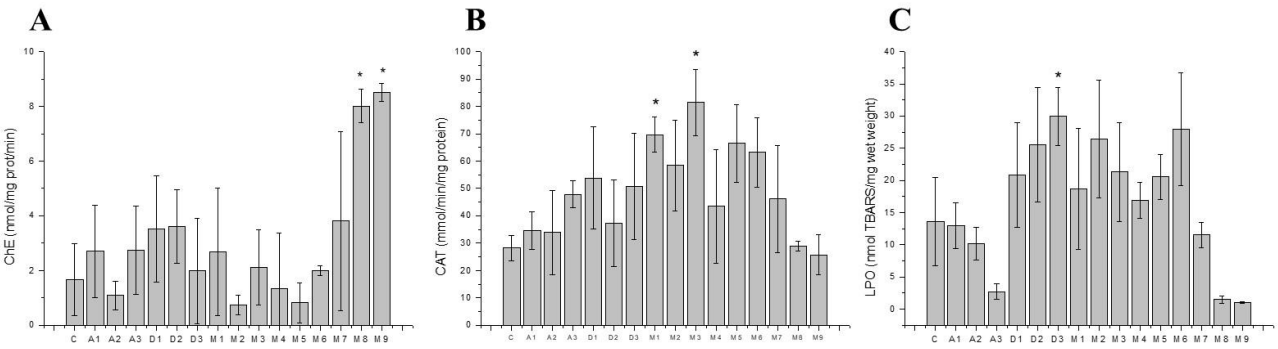

Figure 3. Biomarkers activities (mean ± SD) in *Daphnia magna* exposed to Kraft® 36 EC (a.i. abamectin: A1-3) and Score® 250 EC (a.i. difenoconazole: D1-3) and their mixtures (M1-9): A) Cholinesterase (ChE) activity B) Catalase (CAT) activity and C) Lipid peroxidation (LPO).

Among neuronal enzymes, cholinesterases (ChE) is most commonly used as a biomarker for environmental pollution because of its key role in the hydrolysis of the neurotransmitter acetylcholine in the peripheral and nervous systems (Holth et al., 2008). Recent studies have shown that pesticides have increased the inhibition of acetylcholinesterase (AChE) in aquatic organisms (de Coen and Janssen, 2003; Ferreira et al., 2015), showing the responsiveness of this biomarker to environmental pollution, especially insecticides, in the impairment of cholinesterase activity and/or changes in cellular redox homeostasis, for a wide

variety of species (Whittaker et al., 1982, Solé et al., 2010, Rhee et al., 2013, Gonzalez-Rey and Bebiano, 2014; Ramos et al., 2014).

Although the effect of insecticides on the inhibition of ChE activity in *D. magna* is well known (Barata et al., 2004), in the present study ChE levels were significantly higher for the two major combinations of the insecticide with the fungicide. The increase of ChE activity in *D. magna* exposed to low concentrations of other compounds has already been reported in the literature, such as for metal (cadmium) and insecticide (carbamate) (Jemec et al., 2007a; Printes and Callaghan, 2004). For other crustaceans, ChE induction was also observed, as for *Palaemon serratus* when exposed to the insecticide deltamethrin, in concentrations as low as $6x10^{7}$mg/L (Oliveira et al., 2012). ChE induction has also been demonstrated for the stonefly *Claassenia* sp. and *Hyalella azteca* crustacean exposed to insecticides fenitrothion (0.001 mg/L) and azinphosmethyl (0.005 mg/L), respectively (Day and Scott, 1990).

An increase in ChE can be explained by compensatory mechanisms after interruption of homeostasis (Calabrese and Baldwin, 2003) or as a result of an attempt of the organism to detoxify through the overproduction of esterases (Hyne and Maher, 2003). Andrade et al. (2016) observed an increase in ChE activity after exposure of zebrafish embryos (*Danio rerio*) to carbendazim, in which they related to the mechanisms of apoptosis. Although the exact mechanisms are not yet understood, indirect evidence that ChE participates in the regulation of apoptosis and cell proliferation has already been discussed (Jiang and Zhang, 2008). A similar mechanism related to the mediation of cellular apoptosis could have played a role in the increased ChE activity observed in the present study. Additional studies should be performed to confirm this hypothesis, especially since it is a complex mixture of the formulation of two pesticides (Fig. 3A). Inert ingredients, which act as solvents, emulsifiers, surfactants, etc. (Cox and Surgan 2006) make it difficult to understand specific metabolic effects in the body. Consistent with this, Kroon et al. (2015) reported that estrogenic biomarkers in juvenile barramundi (*Lates calcarifer*) had increased activity after exposure to a commercial mixture of diuron (Diurex® WG) but not after exposure to analytical-grade diuron, suggesting a response to the formulation additives.

Oxidative stress can be measured using various biochemical biomarkers, such as catalase (CAT), an antioxidant enzyme that acts on the removal of H_2O_2, which is metabolized to O_2 and water and against reactive oxygen species (ROS) and is mainly involved in peroxide of hydrogen produced from the metabolism of fatty acids in peroxisomes (Li et al., 2010; Afiyanti and Chen, 2014). The CAT values for *D. magna* in control groups of previous studies showed enzyme activity values of 62.4 μmol/mg/prot/min (after 22 days) and 250

μmol/mg/prot/min (after 6 days) (Barata et al., 2005; Jemec et al., 2007b). In the present study, the levels determined for CAT were slightly lower compared to these reported values. This may be explained by the fact that but several factors can cause variability in this parameter, such as the type of food (eg. species of algae), age and litter of organisms and experimental conditions (for example, temperature or photoperiod).

The response to exposure to pesticide mixtures at lower concentrations (M1 and M3) (Fig. 3B) consisted of an increase in the activity of this antioxidant enzyme when compared to the control, which may indicate a mechanism of response to oxidative stress (Vega and Pizarro, 2000). Following this increase, it was observed that for the other treatments there was no significant difference. This suggests that the enzyme is involved in the protective response by Daphnia's antioxidant systems to counteract the adverse effects of hydrogen peroxide on the specified pesticide combinations with the occurrence of the effect. Other studies have shown that the activity of antioxidant enzymes may increase when the organism is exposed to low concentrations of chemicals or during short-term exposures, but may decrease or be inhibited in high concentration or after prolonged exposure, thus depending on the concentration tested (Valavanidis et al., 2006; Wang et al., 2011).

However, a significant difference in LPO was observed only for the higher concentration of difenoconazole. For abamectin alone and for some mixture combinations (especially M8 and M9) (Fig. 3C), this decrease was concomitant with an increase in the activity of ROS (CAT) cleavage enzymes. It is possible that this resulted from a prompt antioxidant response by the organisms to these concentrations. Vernouillet et al. (2010) observed a decrease in lipid peroxidation when the crustacean *Thamnocephalus platyurus* was exposed to carbamazepine and suggested that carbamazepine preventing fatty acid oxidation in the membranes by acting as a radical scavenger.

For the isolated exposure to difenoconazole the opposite occurred (decreased CAT and increased LPO) in which, effects on LPO were attenuated (Fig. 3C). This seems to indicate an imbalance in the redox balance of organisms to an oxidative stress situation, as previously described in several organisms (including the European eel and collembola) when exposed to port water, carbamazepine, fluoxetine and C60 fullerene nanoparticles (Ahmad et al., 2004). Literature for *D. magna* in relation to LPO data, measuring TBARS is still quite scarce.

7.4. Energy Reserves

No significant effects were not observed on protein content for the treatments made (F = 1,275, p = 0.256) (Figure 4A). For lipid reserves, only the treatment of M9 mixtures (2815.26 mJ/org) showed significant difference in relation to the control (1955, 28 MJ/org) (F = 3,881, $p < 0.05$) (Figure 4B). With regard to energy consumption (EC), all treatments showed a significant approximately three-fold increase as compared to controls (F = 8,603, $p < 0.05$) (Figure 4C).

Although the mean values of protein content in the pesticide treatments were higher than those in controls, no significant difference could be demonstrated (Fig. 4A). Several authors have reported an increase in protein content for *D. magna*, *Danio rerio* and *E. albidus* exposed to lindane, polluted effluents and carbendazim (de Coen and Janssen, 2003a; Novais and Amorim, 2013; Smolders et al., 2003). A hypothesis for this increase is related to the induction of protein synthesis used for detoxification mechanisms (Novais and Amorim, 2013; Smolders et al., 2003).

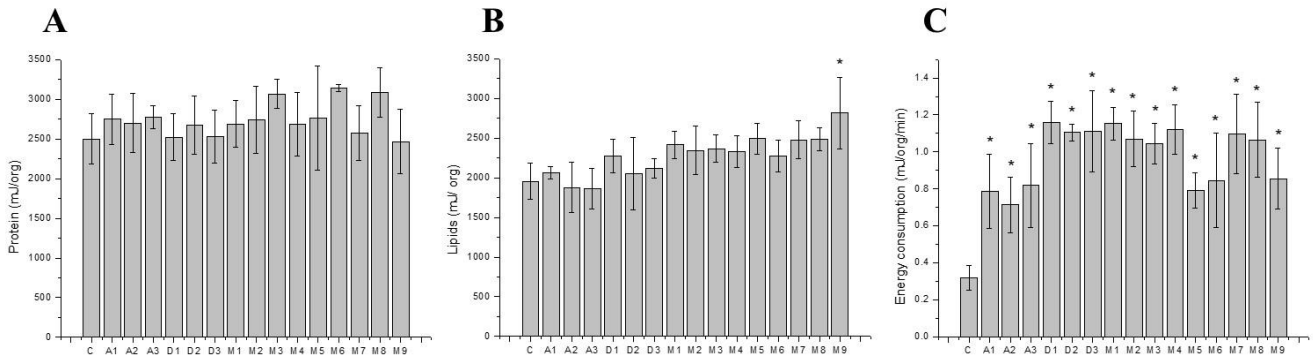

Figure 4. Energy-related parameters (mean ± SE) in *Daphnia magna* exposed to Kraft® 36 EC (a.i. abamectin) and Score® 250 EC (a.i. difenoconazole) and their mixtures: A) Protein contents B) Lipids and C) Energy consumption.

For the lipid reserves, little difference was observed in the treatments except for the highest combination of the pesticide mixtures (Fig. 4B). In crustaceans, lipids serve as membrane building materials and energy storage molecules, and specifically in cladocerans, lipids are also known to be involved in egg production (Goulden and Henry, 1987). Because our exposure was 96 h, the *Daphnia* sp. were preparing for the first egg laying and therefore, the lipid reserves were not affected. Jeon et al. (2013) observed a decrease in lipid content when *D. magna* was exposed for 116 h to the carbamate pesticide carbaryl. Kim et al. (2014) studied the effects of tetracycline on four generations of *D. magna* and throughout the generations, the reductions of the lipid reserves that were initially observed recovered (comparing with the

control group) at a later stage in the experiment, suggesting some adaptation or energy compensation.

The energy activity determined by the electron transport system (ETS) is an inclusive biomarker of the metabolic energy requirements of an organism, since it is directly related to the process of oxygen consumption (Saraiva et al., 2017). Thus, the significant increase in energy consumed (Ec) in all treatments after exposure to insecticide and fungicide and their mixtures (Fig. 4C), reveals high energy demand, hyperactivity related consumption or necessary to deal with detoxification processes.

Rodrigues et al. (2015), when exposing *C. riparius* to low concentrations of the insecticide esfenvalerate, suggested that energy consumption activity (Ec) may be a good indicator of stress and that it also seems to be correlated with effects at higher levels of biological organization. Their increased activity leads to hyperactivity and can also increase detoxification mechanisms, which are processes with high energy requirements.

8. Conclusions

Some changes in molecular biomarkers, reproduction and energy-related parameters in *Daphnia magna* were demonstrated after exposure to sublethal concentrations of abamectin, difenoconazole and their mixtures. The present study demonstrated that the toxic effects on *D. magna* are likely to occur at environmentally realistic exposure levels. However, the actual risk for aquatic life in the field could even be greater than that observed, since higher exposure levels than those evaluated in the present study may be anticipated in the field. In sublethal concentrations of the Kraft® 36 EC and Score® 250 EC and its mixtures, the reproduction of *D. magna* was unaffected. These compounds acting in isolation did not cause effects on cholinesterase (ChE) and Catalase (CAT), but the mixtures caused induction of these biochemical biomarkers. Lipid peroxidation (LPO) was induced when *D. magna* was exposed to low concentration of fungicide. Low concentrations of pesticides acting in isolation and mixtures have not caused changes in protein quantification, but the highest combination of pesticides tested has caused ncreased lipid reserves. The energy consumed was highly affected and stimulated when *D. magna* was exposed to low concentrations of pesticides in isolation and in all combinations. Therefore, prospective environmental risk assessment should take into consideration additional information needed to develop practical criteria for selecting pesticide mixtures, with the aim of reducing the potential risks associated with the ecotoxicity of these potentially highly toxic compounds. The toxic effects of mixtures include aspects of

toxicokinetics and toxicodynamics and therefore it is necessary to understand the underlying toxicity and species detoxification. These mechanisms which may differ in different concentrations of pesticide mixtures and in different test organisms.

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

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Conclusões finais

Com este estudo, as seguintes conclusões gerais puderam ser obtidas:

- Devido ao seu importante papel, diversidade em ecossistemas de água doce tropical e sensibilidade, existe grande potencial para espécies nativas de rotíferos serem utilizadas como organismos-teste em avaliações da toxicidade e de risco de agrotóxicos;

- Efeitos tóxicos sobre *Daphnia magna* ocorrem quando expostas ao inseticida/acaricida Kraft® 36 EC e ao fungicida Score® 250 EC em níveis de exposição ambientalmente realista;

- Em concentrações subletais dos agrotóxicos Kraft® 36 EC e Score® 250 EC e suas misturas, a reprodução de *D. magna* foi pouco afetada;

- Abamectina e difenoconazol agindo de forma isolada não causaram efeitos sobre colisterase (ChE) e catalase (CAT), mas a misturas causaram indução destes biomarcadores bioquímicos;

- Peroxidação lipídica (LPO) foi induzida quando *D. magna* foi exposta a baixa concentração do fungicida;

- Baixas concentrações de Kraft® 36 EC e Score® 250 EC agindo de forma isolada e em misturas não causaram alterações na quantificação de proteínas em *D. magna*, porém a mais elevada combinação dos agrotóxicos testada provocou aumento nas reservas lipídicas;

- A energia consumida é altamente afetada e estimulada quando *D. magna* é exposta a baixas concentrações de abamectina, difenoconazol e suas misturas;

- Os agrotóxicos Kraft® 36 EC (abamectina) e Score® 250 EC (difenoconazol) causam efeitos tóxicos letais ao cladócero Neotropical *Macrothrix flabelligera;*

- Os efeitos tóxicos sobre o cladócero nativo, *M. flabelligera* são susceptíveis de ocorrer em níveis de exposição ambientalmente realistas através do escoamento superficial (*runoff*) e pulverização (*spray drift*);

- Os testes de toxicidade aguda mostraram que as misturas de abamectina e difenoconazol causaram desvios significativos da toxicidade prevista pelo modelo de referência de ação independente (IA), apresentando interações sinérgicas, sendo as interações dependentes do nível da dose (DL);

- *Macrothrix flabelligera* apresentou elevada sensibilidade aos compostos abamectina e difenoconazol. Porém, outros cladóceros têm maior sensibilidade em relação ao inseticida e outras espécies (algas, crustáceos e fungos) para o fungicida.

- Os agrotóxicos abamectina e difenoconazol devem ser avaliados em avaliações de risco não apenas individuais, mas também como misturas.

Considerações finais

Após assumida a importância da utilização de rotíferos em Ecotoxicologia, ressalta-se a necessidade de conduzir mais estudos com diferentes espécies, além da comumente utilizada, *B. calyciflorus*, pois assim, maiores comparações poderão ser realizadas intra e inter-espécies sobre a sensibilidade dos rotíferos aos contaminates em ambiente aquático. Também devem ser ampliados estudos que façam avaliações por meio de testes de toxicidade crônica, sobre bioacumulação e biomagnificação e maiores investimentos em espécies nativas a serem usadas para cenários da região Neotropical.

O presente estudo demonstrou que os efeitos tóxicos sobre *M. flabelligera* são susceptíveis de ocorrer em níveis de exposição ambientalmente realistas. Embora não tenha sido a espécie mais sensível quando comparada a outras espécies da biota aquática, sua sensibilidade pode ser alterada quando exposta a diferentes contaminantes, pois a toxicidade de agrotóxicos formulados podem causar diferentes respostas, tanto devido aos ingredientes "inertes" associados, como pelas respostas de toxicidade serem espécie-específicas. Além de uma sensibilidade possivelmente diferente, o uso de espécies nativas em avaliações de efeitos tropicais é recomendado por várias outras razões, como, por exemplo, serem espécies ecologicamente relevantes, apresentarem disponibilidade imediata e, portanto, com menos restrições logísticas além de se evitar a introdução de espécies exóticas em ecossistemas tropicais.

Este estudo demonstrou que as misturas dos agrotóxicos abamectina e difenoconazol causaram maior toxicidade (sinergismo) ao cladócero *M. flabelligera* do que os compostos isolados. Além disso, para as avaliações em nível molecular, energético e reprodutivo em *Daphnia magna*, efeitos só foram observados quando estes compostos agiram em combinação. Por isso, as avaliações prospectivas de risco ambiental e os critérios de qualidade ambiental baseados em exposições aos compostos individuais podem, não proteger adequadamente a biota dos ecossistemas aquáticos. Embora tenha havido um grande aumento na pesquisa sobre a toxicidade de misturas nos últimos anos, são necessárias informações adicionais sobre os tipos de interações que ocorrem em condições naturais, especialmente para concentrações susceptíveis de ocorrer no campo.

Para os estudos das simulações de escoamento superficial (*runoff*) e pulverização (*spray drift*) foram utilizadas apenas as aplicações simples das doses recomendadas pelo fabricante dos produtos isolados Kraft® 36 EC e Score® 250 EC. Uma vez que o uso excessivo e as práticas agrícolas são comuns em áreas agrícolas brasileiras, o risco real sob perfis de exposição no

campo provavelmente será maior do que os resultados aqui apresentados. Assim, é necessário aumentar esforços para realizar estudos futuros para implementar técnicas analíticas químicas que permitam caracterizar os perfis de exposição dos agrotóxicos no campo e posteriormente compará-los com os resultados das simulações dos modelos de destino de agrotóxicos.

Práticas de manejo que reduzam os riscos potenciais associados à toxicidade dos agrotóxicos abamectina e difenoconazol, que são altamente tóxicos precisam ser desenvolvidas. Por exemplo, o gerenciamento integrado de pragas para reduzir a aplicação repetida, diminuir o tempo de exposição das espécies não-alvo e minimizar o risco de desenvolvimento de resistência a pragas, além das ampliações e implementações de programas educacionais ao público sobre essas práticas.

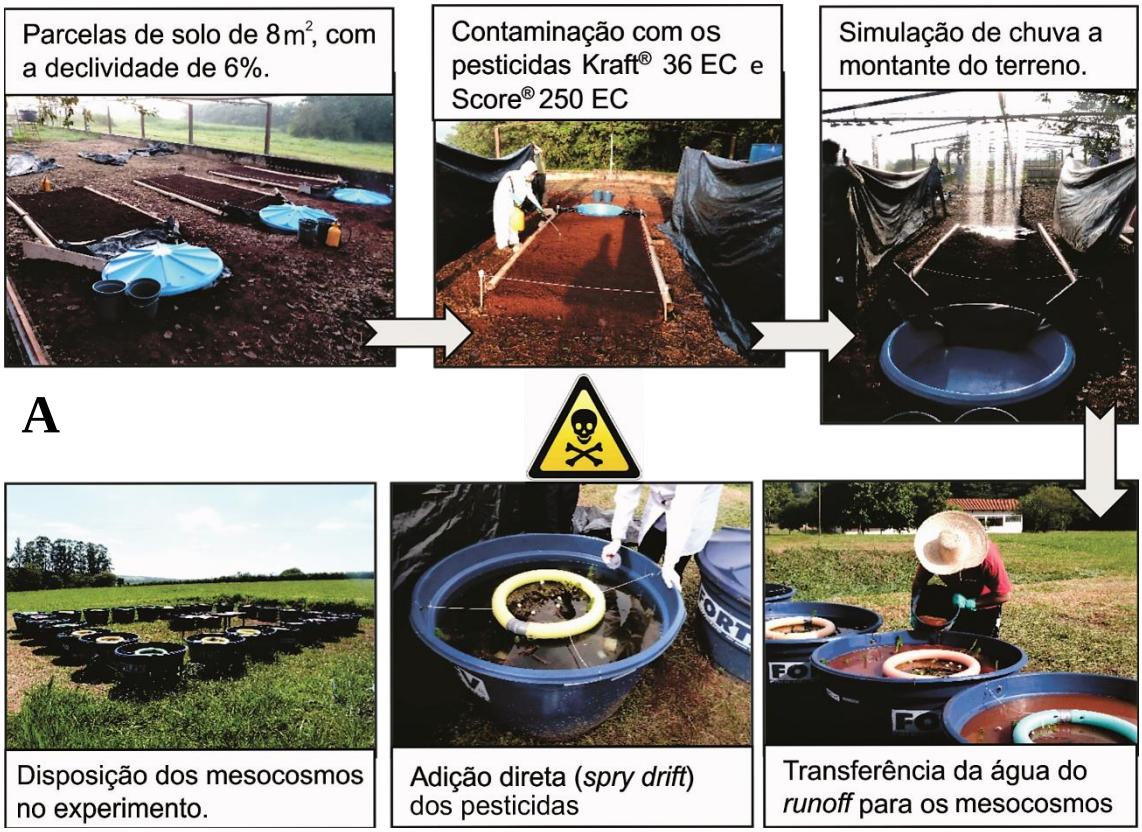
Apêndice
Fotos dos experimentos

Figura 1. Cultivos-estoque de *D.magna* foram mantidos em béqueres de 1 L, sob temperatura controlada de 20 ± 2 °C e fotoperíodo de 16h claro: 8h escuro.

Figura 2. Vista geral da água reconstituída utilizada para a manutenção dos cultivos-estoque das espécies de cladócero *Macrothrix flabelliger*a, preparada de acordo com os procedimentos recomendados pela Associação Brasileira de Normas Técnicas ABNT (2005) e alga clorofícea, *Raphidocelis subcapitata*, utilizada para alimentação dos organismos-teste.

Figura 3. Cultivos-estoque de *M. flabelligera* mantidos em béqueres de 1 L, em incubadoras com temperatura controlada a 25 ± 1º C e fotoperíodo de 12h claro: 12 h escuro. Béqueres lacrados com filme de plástico para evitar a evaporação do meio de cultura.

Figura 4. Experimentos de toxicidade aguda com a espécie *M. flabelligera* mantidos sob a mesma temperatura dos cultivos-estoque, sem alimento e iluminação.



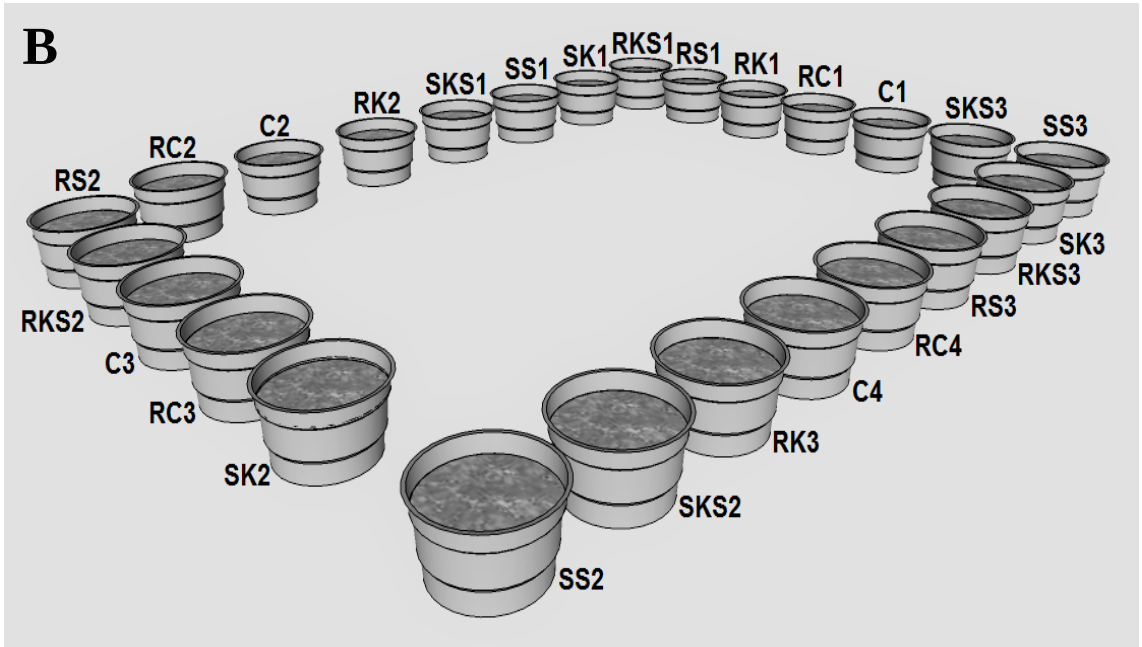


Figura 5. Representação passo a passo da realização da contaminação dos microcosmos (A) e esquema da disposição dos tanques no experimento (B).