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MARTA SILVA MUNIZ

TOXICIDADE AGUDA DA MOXIDECTINA EM EMBRIÕES DE PEIXE-ZEBRA (*Danio rerio*): BIOACUMULAÇÃO E RESPOSTA DE MÚLTIPLOS MARCADORES

UNIVERSIDADE FEDERAL DA PARAÍBA
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JOÃO PESSOA – PB
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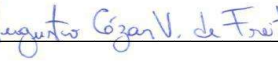

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LISTA DE ABREVIAÇÕES

LMs – Lactonas macrocíclicas

MAPA – Ministério da Agricultura, Pecuária e Abastecimento

VICH – *International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products*, Cooperação Internacional para a Harmonização de Requerimentos Técnicos para o Registro de Produtos Veterinários

Hpf – Horas pós-fertilização

Dpf – Dias pós-fertilização

OECD – *Organisation for Economic Co-operation and Development*, Organização para a Cooperação e Desenvolvimento Econômico

FET – *Fish Embryo Acute Toxicity*, Toxicidade Aguda em Embriões de Peixes

SUMÁRIO

RESUMO

No Brasil, as drogas utilizadas em rebanhos bovinos têm grande potencial contaminante, uma vez que o número de animais no rebanho brasileiro supera o número de habitantes. Dentre estas drogas, os antiparasitários se destacam por serem utilizados de forma contínua e profilática em planos de manejo de pragas. Neste contexto, as lactonas macrocíclicas aparecem como a classe de antiparasitários mais importante no cenário global, abrangendo dois subgrupos: avermectinas e milbemicinas. Embora os efeitos ambientais de várias avermectinas tenha atraído atenção de pesquisadores, estudos sobre os efeitos ambientais da principal milbemicina, a moxidectina, continuam escassos. Portanto, o presente trabalho visou elucidar os potenciais efeitos da moxidectina em embriões de peixe-zebra (*Danio rerio*), numa perspectiva de fornecer informações sobre seu perfil ecotoxicológico. Para isso, foram estudados os seus efeitos apicais, bem como marcadores enzimáticos de neurotransmissão colinérgica, processamento de xenobióticos, estresse oxidative e metabolismo anaeróbico. Também foi estudada a distribuição espacial e a captação do químico pelos embriões de zebrafish por meio de imagens de MALDI-MS e espectrometria de massas, respectivamente. A toxicidade aguda da moxidectina para os embriões de peixe-zebra manifestou-se principalmente como uma diminuição nas taxas de eclosão até 96 hpf (EC_{50} = 18,98 µg/L). Também foi verificado um aumento na atividade enzimática de todos os biomarcadores enzimáticos, além de um acúmulo da droga nos embriões, que apareceu em concentrações de 0,003 a 0,03 µM nas soluções de exposição, contra 50 a 200 µM nos embriões. As imagens de MALDI-MS revelaram acumulação do fármaco principalmente na cabeça e nos olhos dos embriões (72 e 96 hpf). Assim, nossos resultados mostraram que a moxidectina causa estresse oxidativo e um claro padrão de neurotoxicidade aos estágios iniciais da vida do peixe-zebra, demonstrando o potencial risco ambiental da moxidectina para os ecossistemas aquáticos.

Palavras-chaves: antiparasitários, neurotoxicidade, embriotoxicidade, bovinocultura, ecotoxicologia, estresse oxidativo.

ABSTRACT

In Brazil, drugs used in cattle have great pollution potential, as the total number of animals exceeds the number of inhabitants. Among these drugs, antiparasitics stand out because they are routinely and prophylactically used in pest control management strategies. In this context, macrocyclic lactones appear as the most important class of antiparasitics worldwide, encompassing two subgroups: avermectins and milbemycins. Although the environmental effects of several avermectins have attracted the attention of researchers, studies on the effects of the main milbemycin (moxidectin) remain scarce. Thus, the present study aimed to elucidate the potential effects of moxidectin in zebrafish embryos (*Danio rerio*) to provide information on its ecotoxicological profile. For this purpose, we studied the effects of moxidectin through apical endpoints, as well as through enzymatic biomarkers for oxidative stress, xenobiotic processing, anaerobic metabolism, and cholinergic neurotransmission. Moreover, the spatial distribution of moxidectin was determined by means of MALDI-MS imaging, in addition chemical analysis of the embryos and respective exposure solutions. Moxidectin toxicity to zebrafish embryos manifested mainly as a decrease in hatching rates at 96 hpf (EC_{50} = 18.98 µg/L). It also increased the enzymatic activity of all selected biomarkers and accumulated in the embryos, appearing in concentrations ranging from 0.003-0.03 µM in exposure solutions and from 50-200 µM in embryos. MALDI-MS imaging revealed pockets of the drug mainly in the head and eyes of the embryos (72 and 96 hpf). Thus, our results show that moxidectin causes oxidative stress and a clear pattern of neurotoxicity in early life stages of zebrafish, demonstrating the need to prioritize this compound for environmental studies.

Keywords: ecotoxicology, neurotoxicity, embryotoxicity, veterinary pharmaceuticals, antiparasitics, moxidectin, oxidative stress.

CAPÍTULO I
Considerações gerais

1. Introdução

Nas últimas décadas, a crescente preocupação com o custo ambiental das atividades humanas revelou que é necessário conhecer os efeitos dos diversos poluentes presentes no ambiente (HAMSCER e BACHOUR, 2018). No entanto, uma avaliação de risco ambiental robusta dessas substâncias depende do acúmulo e padronização de conhecimentos sobre as diversas variáveis relacionadas ao seu uso, suas propriedades farmacológicas e ecotoxicológicas (DI NICA et al., 2015).

O Brasil é um dos maiores produtores pecuários do mundo, abrigando um rebanho bovino maior que sua população (IBGE, 2019a; IBGEb, 2019b). Assim, fármacos autorizados para utilização em bovinos têm potencial de utilização em larga escala e, consequentemente, de estarem presentes no ambiente e causarem alterações fisiológicas em organismos não-alvo. Em especial, os fármacos que são pouco metabolizados ou de uso tópico chegam ao ambiente sem sofrer alterações, podendo atingir ambientes aquáticos por meio de rotas diversas, inclusive pela deposição direta pelos animais em fontes de água ou por meio da adubação de lavouras com resíduos contaminados (MESA et al., 2018; HAMSCER e BACHOUR, 2018; BOXALL et al., 2003).

Os antiparasitários ocupam uma posição importante no mercado brasileiro de medicamentos veterinários, pois são usados em planos permanentes de manejo de carrapatos, piolhos e helmintos, uma vez que essas pragas causam doenças aos animais e interferem na sua capacidade zootécnica, causando perdas ao produtor (MAQBOOL et al., 2017). Dentre os produtos presentes em formulações para bovinos no Brasil está a moxidectina, uma milbemicina da classe química das lactonas macrocíclicas (LMs). As LMs são uma das famílias de antiparasitários mais utilizadas no mundo, sendo autorizada para o tratamento de parasitos em animais em mais de 60 países (LUMARET et al., 2002). O químico mais representativo das lactonas macrocíclicas é a ivermectina, cujos efeitos ecotoxicológicos são relativamente bem entendidos (BOONSTRA; REICHMAN; VAN DEN BRINK, 2011; BRINKE et al., 2010; LUMARET et al., 2012; MESA et al., 2020). Em contraste, os efeitos das milbemicinas sobre organismos não-alvo são pouco conhecidos. Neste contexto, é importante destacar que as milbemicinas diferem estruturalmente das avermectinas, podendo afetar seu modo de ação e consequentemente os seus efeitos sobre organismos alvo e não-alvo (PRICHARD et al, 2012). Considerando

que a moxidectina é a principal droga no subgrupo das milbemicinas, é urgente a necessidade de caracterização do seu risco ambiental (DI NICA et al., 2015; LUMARET et al., 2012; KIM et al., 2008).

Caracterizar o risco ambiental de poluentes requer, além do conhecimento das possíveis rotas de chegada ao ambiente, uma ampla caracterização dos efeitos que podem ser desencadeados em diversos organismos não-alvo (LAYTON et al., 2015). Nesse contexto, o pequeno peixe de água doce *Danio rerio* é um modelo útil para o entendimento da toxicidade de contaminantes em peixes. Trata-se de um modelo animal de baixo custo e alta fecundidade, possibilitando um escaneamento de média a alta performance para a identificação de toxicantes em potencial. Particularmente, o desenvolvimento embrionário rápido e externo ao organismo da fêmea permite que este processo seja observado facilmente, sendo especialmente relevante para estudos de toxicidade embrionária (BAMBINO e CHU, 2016; KOENIG, KAN e SHIH, 2016).

Assim, o presente estudo busca elucidar os efeitos da exposição de embriões de peixe-zebra (*D. rerio*) à moxidectina, a fim de fornecer informações de cunho ecotoxicológico que sejam úteis à avaliação de seus riscos ambientais.

2. Fundamentação teórica

2.1. Produtos de uso veterinário e implicações ambientais

É crescente a preocupação com os efeitos ambientais causados por poluentes emergentes como fármacos e cosméticos (AWFA et al., 2018). Entretanto, o interesse inicial se concentrou nos riscos gerados por substâncias de uso humano. Mais tarde, substâncias de uso veterinário surgem como poluentes emergentes, uma vez que são usados mundialmente em sistemas pecuários de forma terapêutica e não-terapêutica (por exemplo, como promotores de crescimento), gerando grandes quantidades de resíduos que chegam ao ambiente por meio da urina, das fezes e da própria aplicação de medicamentos tópicos por aspersão ou banho carrapaticida – práticas amplamente utilizadas no manejo de carrapatos em rebanhos de ruminantes no mundo todo (YOPASÁ-ARENAS e FOSTIER, 2018; SINDAN, 2019; ASLAM et al., 2018; BOXALL et al., 2003). Assim, as substâncias de uso veterinário têm recebido mais atenção, com destaque para resíduos de antibióticos provenientes de criações muito intensivas, como as produções aviárias e suínas que geram grande quantidade de resíduos

com altas concentrações de biocidas (HAMSCER e BACHOUR, 2018). Entretanto, outras substâncias de uso frequente, como os antiparasitários e aditivos, também podem oferecer riscos ambientais distintos em criações intensivas e extensivas (KOOLS et al., 2008; KOSCHORRECK, KOCH e RÖNNEFAHRT, 2002).

As informações mais recentes do governo brasileiro revelam um rebanho de cerca de 213,5 milhões de cabeças de gado bovino no país em 2018, contra uma população brasileira projetada em aproximadamente 210,1 milhões de pessoas (IBGEa, 2019; IBGEb, 2019), o que evidencia a escala do uso de fármacos de uso médico-veterinário no país. Na produção de ruminantes, o uso de fármacos antiparasitários é constante, uma vez que as estratégias de controle de parasitoses são ações contínuas, devendo ser cuidadosamente planejadas e implementadas para garantir uma produção sustentável a longo prazo (DONIZETE, SOUZA e BAFFI, 2010).

Assim, os antiparasitários são utilizados extensivamente na pecuária brasileira e estão constantemente presentes no ecossistema agropastoril, podendo ainda atingir os ecossistemas aquáticos adjacentes rotas diversas que incluem: (1) sendo depositados no solo durante a aplicação de medicamentos via tópica por aspersão ou banho carrapaticida; (2) sendo excretados, junto com seus metabólitos, nas fezes e urina, que são então utilizados como adubo e ganham potencial de lixiviação; (3) sendo depositadas diretamente pelos animais em fontes de água nas fezes, urina ou resíduos do medicamento após aplicacao por via tópica e; (4) por inundações sazonais em certos ecossistemas (MESA et al., 2018; HAMSCER e BACHOUR, 2018; BOXALL et al., 2003). A primeira rota pode também ocorrer quando os animais são criados de forma extensiva às margens de rios ou em áreas inundáveis (MESA et al., 2018; YOPASÁ-ARENAS e FOSTIER, 2018).

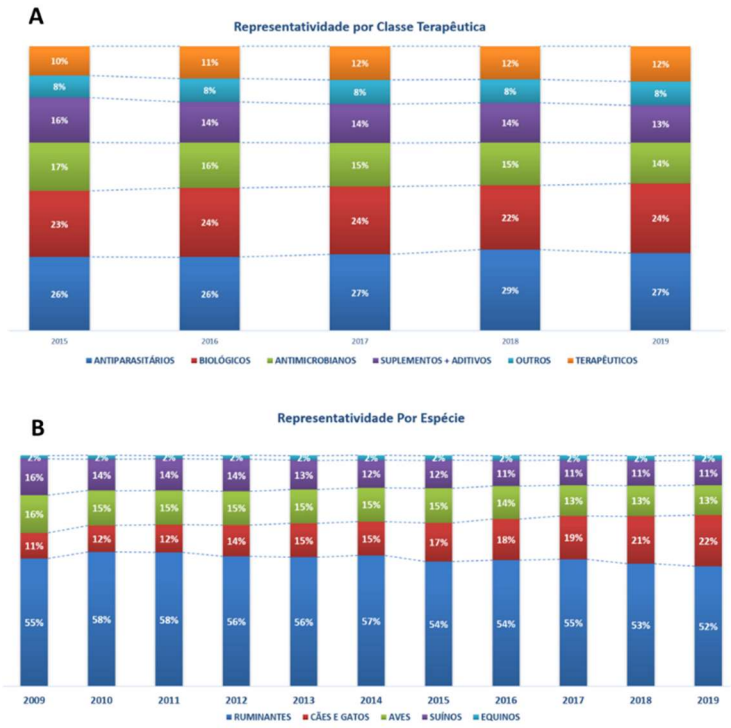
O licenciamento e a renovação de anti-helmínticos para uso veterinário são regidos pela portaria Nº 48, de 12 de maio de 1997 do Ministério da Agricultura (MAPA, 1997a), que preconiza uma série de testes de eficácia e segurança para o hospedeiro, além da reavaliação do registro a cada dez anos. Entretanto, a portaria mas não faz considerações de cunho ambiental, como o requerimento de avaliação de risco ambiental. Além disso, a compra dessas substâncias é frequentemente efetuada de forma irrestrita e sem prescrição do médico veterinário, servindo como fator catalisador dos possíveis riscos ambientais inerentes ao uso de biocidas agropecuários (DONIZETE; SOUZA; BAFFI, 2018).

2.2. Riscos ecológicos de antiparasitários de uso veterinário

Do ponto de vista ecológico, a preocupação mais imediata é a interferência de resíduos de antiparasitários no ecossistema agropastoril. Como a eliminação de muitos antiparasitários e seus metabólitos ocorre pelas fezes e urina, as comunidades de insetos que colonizam o esterco ficam imediatamente vulneráveis, o que tem levado a um maior interesse em entender os possíveis impactos ecológicos e produtivos do manejo de pragas na pecuária, bem como formas mais sustentáveis de manejo de verminoses em ruminantes, como controle integrado, que busca minimizar o uso de agentes químicos por meio da integração de diferentes estratégias, incluindo pastejo rotacionado e uso de controle biológico (DONIZETE; SOUZA; BAFFI, 2018; LUMARET et al., 2012).

Para além do sistema agropastoril, esses medicamentos podem ainda chegar a ambientes aquáticos, onde podem afetar direta ou indiretamente os organismos nestes ambientes ao longo da cadeia trófica (PUFAL et al., 2019). Em termos de potencial de poluição ambiental, os antiparasitários destinados a ruminantes ocupam uma posição crítica, sendo os produtos de saúde animal mais comercializados no Brasil (Figura 1), uma vez que sua utilização profilática e rotineira é indispensável para a manutenção de animais de alta produção e baixo custo (SINDAN, 2019).

Figura 1 – Posicionamento dos antiparasitários de uso veterinário no mercado brasileiro em 2019.



Fonte: SINDAN, 2019.

2.3. Avaliação de risco ambiental de antiparasitários de uso veterinário

No Brasil, a enormidade do rebanho bovino e seus diversos regimes de produção (extensiva, semi-extensiva e intensiva) são fatores que se relacionam com ambas as rotas de dissipação de fármacos no meio ambiente (YOPASÁ-ARENAS e FOSTIER, 2018; DI NICA et al., 2015). Porém, a concretização dessa poluição depende de muitas variáveis, incluindo a aderência da substância ao solo e sua estabilidade no meio ambiente (PEREIRA et al., 2012). Do ponto de vista da avaliação de risco, é necessária a demonstração não somente da probabilidade de ocorrência do fármaco no meio ambiente, mas também a caracterização de um eventual dano, para que então o risco possa ser classificado de acordo com a relação entre o perigo e o dano (LAYTON et al., 2015). Assim, além do potencial de poluição ambiental, os efeitos deletérios de um determinado fármaco devem também ser caracterizados.

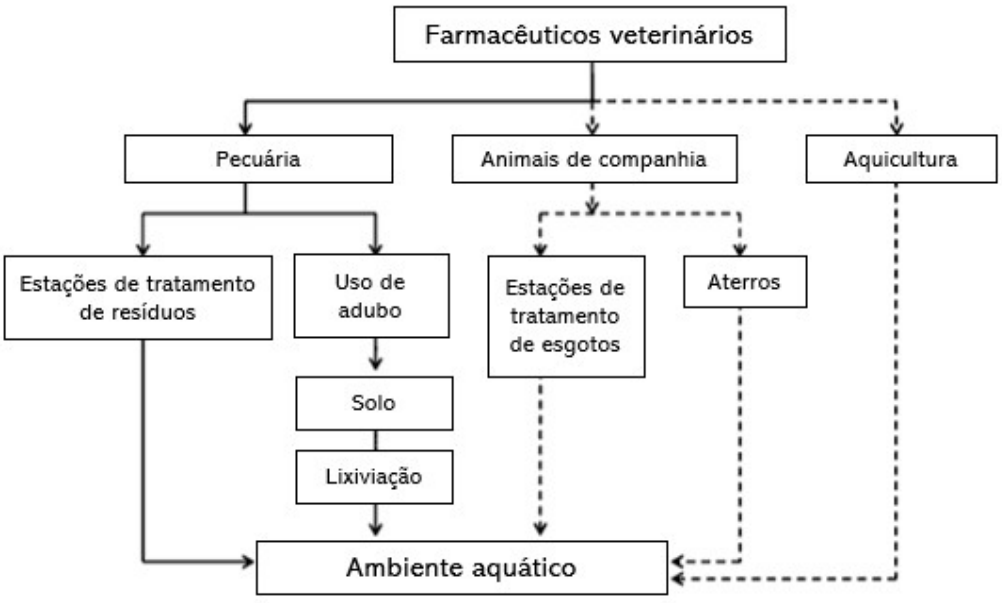
A caracterização do dano em um determinado ecossistema é complexa por natureza, tanto pelo número de variáveis que influenciam a dissipação e a estabilidade dos químicos no ambiente quanto pelo número de organismos não-alvo que podem ser atingidos. Além disso, um mesmo fármaco pode desencadear efeitos distintos em diferentes taxa, sendo necessária a caracterização dos possíveis danos decorrentes do contato com um fármaco em vários organismos vivos ao longo da cadeia trófica (PUFAL et al., 2019; BIAŁK-BIELIŃSKA, 2011). No caso dos antiparasitários de uso veterinário, são muito escassas as informações cruciais acerca de muitos fármacos, como a taxa de absorção pelo solo e detecção de fármacos em reservatórios naturais de água (DI NICA et al., 2015).

Os esforços para elucidação dos riscos ambientais oferecidos por medicamentos de uso veterinário são relativamente recentes, e as lacunas são consideráveis. Tentativas de montar *rankings* de priorização desses fármacos para ambientes aquáticos diferem em metodologia, abrangência e critérios de classificação (DI NICA et al., 2015; KIM et al., 2008; KOOLS et al., 2008). Na mais recente e mais compreensiva delas, Di Nica et al. (2015) propuseram que fossem utilizados os critérios previamente recomendados pela Cooperação Internacional para Harmonização dos Requerimentos Técnicos para Registro de Produtos Veterinários (VICH, do inglês *International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products*), numa tentativa de padronizar os estudos de priorização de fármacos veterinários para ecossistemas terrestres

e aquáticos. Os autores esclarecem ainda que a falta de informações sobre essas drogas foi uma das maiores dificuldades encontradas para a concretização do estudo.

Alguns critérios podem ser estabelecidos para a classificação sistemática de medicamentos veterinários, dos quais o mais proeminente e acessível é a popularidade do princípio ativo, que é medida com base na quantidade comercializada em um dado território. Entretanto, não há no Brasil um sistema de divulgação unificado e confiável de dados sobre a comercialização de antiparasitários de uso veterinário, embora a resistência parasitária seja um fenômeno extensivamente documentado e os resíduos destes fármacos já tenham sido priorizados para monitoramento em produtos de origem animal destinados ao consumo humano (ANVISA, 2018; DONIZETE; SOUZA; BAFI, 2010), o que evidencia a escala do uso dessas substâncias. Outros critérios, como espécie-alvo, taxa de metabolização e via de administração são considerados indicadores importantes do risco ambiental, de forma que Kim et al. (2008) e Di Nica et al. (2015) concordam que substâncias aprovadas para uso tópico em bovinos oferecem maior risco ao ambiente, uma vez que o medicamento não é metabolizado, adentrando o ecossistema em sua forma intacta (Figura 2).

Figura 2 – Rotas de poluição de ambientes aquáticos por farmacêuticos de uso veterinário. Linhas contínuas representam rotas de maior importância, em comparação às rotas de linhas pontilhadas.



Fonte: adaptado de Kim et al. (2008)

Adicionalmente, é preciso enfatizar que o uso de antiparasitários na produção de ruminantes é contínuo, fazendo parte de um sistema preventivo e remediativo de manejo

de carrapatos, moscas e endoparasitos que causam sérias perdas econômicas ao produtor e podem tornar os animais susceptíveis a enfermidades mais graves (MAQBOOL et al., 2017). Idealmente, esse manejo deve ser acompanhado pelo médico veterinário e incluir medidas que vão além do controle químico, como o pastejo rotacionado e o controle biológico, a fim de reduzir as intervenções medicamentosas (COSTA; SIMOES; RIET-CORREA, 2011). Na realidade, porém, o uso indiscriminado de antiparasitários é uma constante no Brasil, aumentando o risco do uso excessivo de antiparasitários e do uso prolongado do mesmo princípio ativo, o que pode, por sua vez, aumentar o potencial de poluição ambiental (DONIZETE; SOUZA; BAFI, 2010).

Assim, é urgente a tarefa de obter informações sobre os possíveis riscos ambientais oferecidos por medicamentos de uso veterinário, para que sejam priorizados e monitorados de acordo com uma avaliação de risco ambiental sólida. Como uma contribuição para o preenchimento dessa lacuna, no Capítulo II é apresentado um artigo de revisão (não publicado) que foi desenvolvido em paralelo ao objetivo geral desta dissertação, a fim de mostrar um painel compreensivo sobre os efeitos de antiparasitários de uso médico-veterinário aos ecossistemas aquáticos, enfatizando estudos de toxicidade para organismos não-alvo. Esse trabalho evidencia o foco dos estudos em organismos invertebrados, a carência de estudos de dissipação e propriedades ambientais de diversos compostos, bem como a predominância de classes químicas importantes como as lactonas macrocíclicas e os benzimidazóis.

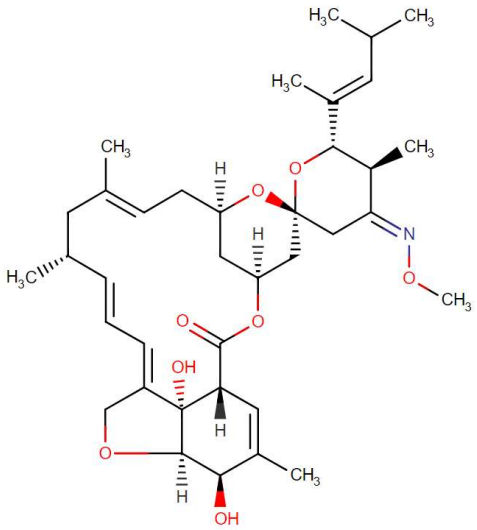
2.4. Lactonas macrocíclicas e moxidectina

Dentre as substâncias registradas pelo MAPA para controle de pragas bovinas no Brasil estão as lactonas macrocíclicas - LMs (MAPA, 2014), família de fármacos antiparasitários que inclui as avermectinas e as milbemicinas. As LMs agem sobre canais iônicos de diversos invertebrados, causando sua paralisia e eventual morte (Prichard et al. 2012). Embora não haja uma divulgação oficial no Brasil de estatísticas sobre o uso desses medicamentos, a moxidectina é um medicamento previamente considerado de alto risco em outros países (DI NICA et al., 2015; BOXALL et al., 2005).

A moxidectina pertence ao grupo químico das LMs, subgrupo milbemicinas (PRICHARD, MÉNEZ; LESPINE, 2012). Trata-se de uma droga endodecticida (com efeito sobre endoparasitos e ectoparasitos) de amplo espectro de ação que é capaz de ligar-se a canais ionicos de cloro essenciais ao funcionamento de músculos e nervos em invertebrados, cusando paralisia fácida (COBB; BOECKH, 2009; PRICHARD; MÉNEZ;

LESPINE, 2012). Estruturalmente, a moxidectina difere das demais MLs por apresentar uma ligação olefinica na posição C-25 da cadeia lateral, um grupo metiloxima na posição C-23 e ausência de um grupamento dissacarídeo na posição C-13 (COBB; BOECKH, 2009) (Figura 3).

Figura 3 – Estrutura química da moxidectina.



Fonte: DrugBank (drugbank.com/drugs/DB11431)

Acredita-se que as LMs sejam os antiparasitários mais utilizados no mundo na prática veterinária, sendo autorizadas em mais de 60 países, além de serem populares por oferecerem baixa toxicidade e proteção de amplo espectro contra parasitos gastrointestinais (LUMARET et al., 2002). O composto mais representativo desse grupo é a ivermectina, cujos efeitos ambientais são os mais bem caracterizados (BOONSTRA; REICHMAN; VAN DEN BRINK, 2011; BRINKE et al., 2010; LUMARET et al., 2012; MESA et al., 2020). Junto a outros compostos, como a doramectina e a abamectina, a ivermectina é considerada de alto risco ambiental pela sua alta toxicidade a organismos aquáticos não-alvo e pela sua alta frequência de uso (LUMARET et al., 2002; KOOLS et al., 2008). As milbemicinas são em geral menos tóxicas, mas sua variação estrutural sugere que seus efeitos podem diferir daqueles causados por outras LMs nos organismos não-alvo, podendo não ser possível extrapolar resultados de ecotoxicidade (PRICHARD, MÉNEZ e LESPINE, 2012). Nos Estados Unidos, a moxidectina foi aprovada para comercialização depois de considerações ecotoxicológicas, mas demonstrou vários níveis de toxicidade a organismos não-alvo, sendo mais tóxica para peixes em comparação com invertebrados (FORT DODGE ANIMAL HEALTH, 1997). Além de apresentar alto risco de ocorrência em ambientes aquáticos, a droga foi classificada na União Europeia como

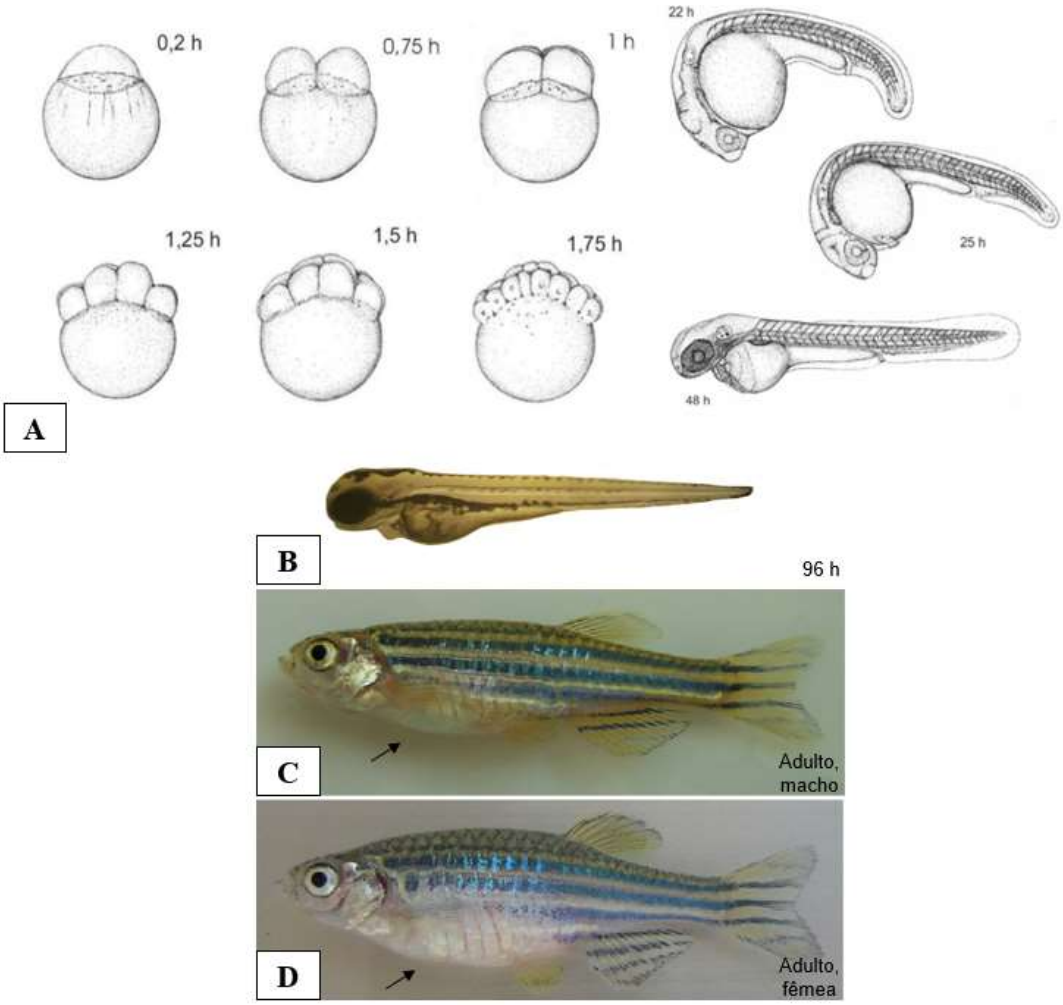
persistente e bioacumulativa em peixes (HEALTH PRODUCTS REGULATORY AUTHORITY, 2018; KOOLS et al., 2008; AGÊNCIA EUROPEIA DE MEDICAMENTOS, 2001).

2.5. Peixe-zebra como modelo para ecotoxicologia de vertebrados aquáticos

O peixe-zebra (*D. rerio*), também conhecido como paulistinha, é um peixe tropical teleósteo já estabelecido como modelo animal para pesquisas biomédicas. Seus embriões de fácil manipulação, oferecendo uma alternativa ao uso intenso de mamíferos e atendendo a uma preocupação bioética que é crescente no Brasil e no mundo (TRIGUEIRO et al., 2020; ABREU et al., 2019).

Como modelo experimental, *D. rerio* oferece vantagens de caráter prático e econômico, uma vez que é um animal de baixo custo de manutenção e alta fecundidade, o que lhe confere um potencial de uso em técnicas de médio e alto desempenho, como o escaneamento de novas drogas e tóxicos em potencial (ABREU et al., 2019; KOENIG, KAN e SHIH, 2016). Além disso, seu ciclo de vida curto permite que os embriões alcancem o estado larval em 48-72 horas pós fertilização (hpf) e se tornem sexualmente maduros quando alcançam em torno de 10 a 12 semanas de vida, o que facilita estudos de natureza crônica ou que envolvem mais de uma geração (MEYERS, 2018). Em estudos toxicológicos, o desenvolvimento embrionário rápido (figura 3), o tamanho da larva e o alto número de indivíduos permitem estudos de alto desempenho para *screening* de toxicidade num organismo inteiro, oferecendo uma visão mais panorâmica dos efeitos tóxicos do que seria possível em culturas celulares. Adicionalmente, os embriões são translúcidos e seu desenvolvimento ocorre fora do organismo materno, permitindo a observação de alterações morfológicas desde as primeiras horas após a fertilização (BAMBINO e CHU, 2016; KOENIG, KAN e SHIH, 2016).

Figura 4 – Estágios do desenvolvimento inicial de *Danio rerio* até as 96 hpf (A, B); mais adultos macho (C) e fêmea (D), distinguíveis por meio do abaulamento da região ventral (setas).



Fontes: A – OECD, 2013; B – realização própria; C, D – adaptado de AVDESH, et al. 2012.

Apesar das muitas vantagens que acompanham o uso do peixe-zebra em estudos toxicológicos, o modelo traz importantes limitações, como as dificuldades de controle sobre as vias de aplicação ou dose dos químicos estudados (BAMBINO; CHU, 2016). Mesmo assim, como o peixe-zebra é um modelo biológico tradicional, muitas informações relevantes já estão disponíveis, como o sequenciamento de seu genoma, linhagens específicas, informações sobre funções fisiopatológicas, assim como a conservação de muitas vias em vertebrados, apresentando inclusive uma ortologia de 70% com o genoma humano (HOWE et al., 2013). Do ponto de vista ecotoxicológico, essas informações facilitam o uso desse modelo biológico como forma de avaliar ao potencial tóxico de poluentes tradicionais e emergentes para vertebrados aquáticos, além de informar sobre uma possível toxicidade para seres humanos (BAMBINO; CHU, 2016).

3. Objetivos

3.1. Geral:

- Avaliar se a moxidectina causa efeitos adversos aos estágios embriolarvais de peixe-zebra (*D. rerio*), numa perspectiva de contribuir para o conhecimento acerca dos efeitos ambientais de antiparasitários de uso veterinário.

3.2. Específicos:

- Avaliar o potencial efeito tóxico agudo da moxidectina em embriões de peixe-zebra;
- Descrever possíveis mudanças fenotípicas e comportamentais decorrentes da exposição à moxidectina;
- Mensurar a capacidade de geração de estresse oxidativo e neurotoxicidade por meio de biomarcadores enzimáticos em embriões do peixe-zebra;
- Determinar a distribuição e o potencial de acumulação da moxidectina em embriões de peixe-zebra;
- Caracterizar a literatura corrente sobre os efeitos ambientais de antiparasitários de uso veterinário por meio de revisão sistemática.

CAPÍTULO II

Artigo I (experimental)

Manuscrito submetido ao periódico *Environmental Pollution* (ISSN: 0269-7491, Elsevier Publishing, fator de impacto: 6.792)

Título: “moxidectin elicits a pattern of neurotoxicity and oxidative stress while accumulating in the head and eyes of zebrafish embryos”

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Moxidectin elicits a pattern of neurotoxicity and oxidative stress while accumulating in the head and eyes of zebrafish embryos

List of abbreviations:

MLs – Macrocyclic lactones

Hpf – Hours post-fertilization

Dpf – Days post-fertilization

DMSO – Dimethyl sulfoxide

OECD – Organisation for Economic Co-operation and Development

FET – Fish Embryo Acute Toxicity

AChE – Acetylcholinesterase

GST – Glutathione S-transferase

CAT – Catalase

LDH – Lactate dehydrogenase

MALDI-MS – Matrix-assisted laser desorption/ionization - Mass Spectrometry

BCF – Bioconcentration factor

BBB – Blood-brain barrier

Highlights

1. Embryos were unable to hatch by 96 hpf (EC_{50} = 18.98 µg/L) after moxidectin exposure
2. Moxidectin induces changes in the activity of biomarker enzymes linked to oxidative stress and neurotoxicity
3. Moxidectin strongly enriched in the embryo compared to the exposure media
4. MALDI-MS imaging detected moxidectin in the head and eye regions of the embryos

Abstract

Moxidectin is an antiparasitic drug belonging to the class of the macrocyclic lactones, subgroup mylbemicins. It is used worldwide in veterinary practice, but little is known about its potential environmental risks. Thus, we used the zebrafish embryo as a model system to study the potential effects of moxidectin on aquatic non-target organisms. The analyses were performed in two experimental sets: (1) toxicity and apical endpoints were characterized, while biomarker assays provided information on the activity levels of catalase, glutathione S-transferase, lactate dehydrogenase, and acetylcholinesterase; and (2) internal concentration and spatial distribution of moxidectin were determined using ultraperformance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-QToF-MS) and matrix-assisted laser desorption/ionization-MS imaging (MALDI-MSi). The acute toxicity to zebrafish embryos (96 hpf) appeared mainly as a decrease in hatching rates (EC_{50} = 18.98 µg/L). It also increased the enzymatic activity of all selected biomarkers and strongly accumulated in the embryos, as internal concentrations were 4 orders of magnitude higher than those detected in exposure solutions. MALDI-MSi revealed accumulations of the drug mainly in the head and eyes of the embryos (72 and 96 hpf). Thus, our results show that exposure to moxidectin causes oxidative stress and a clear pattern of neurotoxicity in the early life stages of zebrafish, demonstrating the need to prioritize this compound for environmental studies.

Keywords: aquatic toxicology, veterinary antiparasitics, emerging contaminants, MALDI-MS, oxidative stress.

1. Introduction

Veterinary antiparasitics are emerging pollutants. They are routinely used in farms around the globe to treat and prevent disease in several species, especially those in grazing systems (Kools et al. 2008; Kim et al. 2008; Di Nica et al. 2015). These chemicals obviously play an important role in animal welfare and food security, but are likely to pose a higher risk to aquatic life than antibiotics (Kools et al. 2008; Carlsson et al. 2013; Kołodziejska et al. 2013; Di Nica et al. 2015; Bundschuh et al. 2016). The preventive nature of its use may drive an insidious presence of these compounds in the ecosystems surrounding agricultural land, warranting detailed understanding of their environmental risks. In cattle farming, they can reach adjacent aquatic ecosystems through two routes: (1) being deposited in the soil during the application of drugs via topical sprinkler or bath; (2) being excreted, along with its metabolites, in feces and urine, which are then used as fertilizer and gain leaching potential (Boxall et al. 2003). The latter route is especially important when animals are raised extensively along the banks of rivers or in flooded areas (Mesa et al. 2017, 2018, 2020; Yopasá-Arenas and Fostier 2018).

Moxidectin is a veterinary antiparasitic belonging to the chemical group of macrocyclic lactones (MLs), and represents the most important compound in the subgroup of the milbemycins (Prichard et al. 2012). MLs are believed to be the most widely used antiparasitic agents in the world in veterinary practice, being authorized in more than 60 countries and having their popularity attributed to the fact that they offer broad-spectrum protection and low toxicity to the host (Lumaret et al. 2012). The most representative compound in this group is ivermectin, whose environmental effects are relatively well characterized. Along with other MLs, such as doramectin and abamectin, ivermectin is considered to be of high environmental risk due to its high toxicity to non-target organisms and high frequency of use (Kools et al. 2008; Lumaret et al. 2012).

Milbemycins possess structural variations that may impact their mode of action, suggesting that their effects may differ from those caused by other MLs, and that it may not be possible to extrapolate results from other MLs to milbemycins (Prichard et al. 2012). In the United States, moxidectin was approved with mitigation requirements, as it showed varying degrees of toxicity to non-target organisms, being more toxic to fish than to invertebrates (Fort Dodge Animal Health 1997). In addition to presenting a high risk of occurrence in aquatic environments, the drug was classified by the European Union as persistent in the environment and bioaccumulative in fish (Kools et al. 2008; European Medicines Agency 2017; Health Products Regulatory Authority 2019). Despite mounting evidence pointing to the need for prioritization of moxidectin, research on the environmental effects of this frequently used mylbemicin is very limited (Prichard et al. 2012; Hentz et al. 2019).

Considering the gaps in hazard characterization of moxidectin, particularly when it comes to its effect in aquatic organisms, we aimed to contribute to the understanding of the environmental impact of this drug by investigating its toxic effects on early life stages of zebrafish. To this end, we investigated the acute toxicity of moxidectin through apical endpoints and enzymatic biomarkers, as well as its internal concentration and spatial distribution within the embryos. To the best of our knowledge, this is the first report on the effects of moxidectin on zebrafish embryos.

2. Materials and Methods

2.1. Chemicals

Moxidectin (CAS No. 113507-06-5) was supplied by AK Scientific (Union City, California, USA) and Sigma-Aldrich (Germany). The matrix α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Sigma-Aldrich (Germany). Moxidectin was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany) to

prepare stock solutions that were stored at -18 ºC. All chemicals and solvents were of analytical grade.

2.2. Animals

The experiments for morphological and biochemical analyses were approved by the Ethics Committee on the Use of Animals at UFPB, with authorization documented by the protocol No. 8881290419. Adult wild-type animals were kept in mixed groups (with males and females) in tanks with a water recirculation system, at a temperature of 26 ± 1ºC and a photoperiod of 14:10h light/dark. After spawning, the embryos (approximately 1 hpf) were washed, cultured in adapted embryonic medium E3 (5.0 mM NaCl, 0.17 mM KCl, 0.33mM CaCl, and 0.33mM MgSO4) and analyzed under an inverted light microscope (Televal 31, Zeiss, Germany) for selection based on developmental stage and viability. Selected embryos were distributed in 96-well microtiter plates, where they were incubated with moxidectin, DMSO, or E3 medium for exposure assays.

For exposure experiments for internal concentration and spatial distribution, embryos were obtained from adults of the OBI-UFZ lineage (*Danio rerio*, generation F16). The adults were originally obtained through a local breeder, then kept for several generations at the UFZ facilities (Fetter et al. 2015). Animals were kept and used according to the animal protection standards of Germany and the European Union, with procedures approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75-9185.64).

2.3. Acute toxicity and biomarker analysis

2.3.1. Exposure assays

Exposure assays were performed according to OECD 236 (OECD 2013). The embryos (≈3 hpf) were distributed individually in 96-well plates where they were exposed to successive dilutions of moxidectin stocks in E3 medium (DMSO = 0.01%, low water solubility (Lanusse et al. 1997) up to the concentration of 20 mg/L. Every 24 h, embryos were observed under an inverted light microscope (Televal 31, Zeiss, Germany) to verify the appearance of lethal and sub-lethal endpoints, for a total period of 96 h. Additionally, because of the short half-life of moxidectin (6.8 h; Fort Dodge Animal Health, 1997), the exposure media was renewed every 24 h, including the controls.

2.3.2. Spontaneous movements and heart rate

Spontaneous movements or coils are unprovoked motor responses observable in embryos between 17 and 27 hpf (Saint-Amant and Drapeau 1998), and changes in the frequency of these movements are thought to be early behavioral manifestations of neurotoxicity (Weichert et al. 2017; Ogungbemi et al. 2019, 2020; Tao et al. 2020), while heart rates may provide insight into cardiovascular alterations at concentrations below those that cause morphological changes (Fraysse et al. 2006; Sun and Liu 2017; Cheng et al. 2020). Ten embryos were exposed to three concentrations below the EC_{50} at which no effect was observed (1.25, 2.5 and 5 µg/L) in 96-well microtiter plates, in the same conditions as described in item 2.3.1. Under a light microscope, the spontaneous movements of individual embryos were observed for 1 min at 24 hpf (± 1 h). At 48 hpf (± 1 h), the heartbeats were observed for another 1 min.

2.3.4. Enzymatic activity assays

As a general depiction of the toxicity induced by moxidectin, we measured the enzymatic activity of classic biomarkers related to: (i) antioxidant response – catalase (CAT) and glutathione S-transferase (GST); (ii) neurotransmission – acetylcholinesterase

(AChE); and (iii) metabolism – lactate dehydrogenase (LDH). Embryos were exposed to three concentrations below the EC_{50} at which no effect was observed (1.25, 2.5, and 5 µg/L). Then, pools of eight organisms were transferred to microtubes containing 0.8 mL of sodium phosphate buffer solution (0.1 M, pH 7.4) and quickly frozen, then stored at -4ºC.

Determination of enzymatic activity was carried out following the procedures described by Rivero-Wendt et al. (2016). The stored samples were thawed in an ice bath, homogenized in phosphate buffer (0.1 M, pH 7.4) and centrifuged for 20 min at 11500 *g* so that the post mitochondrial supernatant is used.

To measure AChE, acetylcholine was used as a substrate. 50 µL of the protein homogenate from the larvae extract were incubated with 250 µL of reaction solution (0.075 M acetylcholine and 0.01 M 5.5'-acid dithiobis- [2-nitrobenzoic] [DTNB]) for 5 min, at 25 ºC. The formation of thiocholine, a degradation product of acetylcholine, was determined with a spectrophotometer (Multiskan G0, Thermo Fischer, USA) at 414 nm, according to the method of Ellman et al. (1961).

The quantification of GST activity was used as an indicator of detoxification capacity, since GST catalyzes the conjugation of the reduced thiol group of glutathione to electrophilic xenobiotics. The test was carried out according to the methodology previously described by Habig and Jakoby (1981), using 100 µL of larvae extract and 200 µL of reaction solution (10 mM reduced glutathione and 60 mM 1-chloro-2,4-dinitrobenzene, CDNB). The absorbance was measured at 340 nm every 20 s for 5 min.

To assess CAT activity, 15 µL of the extract were added to 135 µL of a reaction solution containing hydrogen peroxide (30 mM) and 150 µL of phosphate buffer

(0.05 mM). The absorbance of the mixture at 240 nm was monitored every 10 s, for 2 min, according to the methodology of Claiborne (1986).

2.4. Chemical analysis and spatial distribution

2.4.1. Exposure assays

Moxidectin was first dissolved in DMSO and then diluted to 640 µg/L (<0.01% DMSO) in ISO water (ISO 2007) in order to reach internal concentrations in the embryos that were sufficient for the ultraperformance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-QToF-MS) analysis. Groups of nine embryos (≥ 2 hpf) were exposed in 18 mL in glass vials for 24, 48, 72 and 96 h. Incubation occurred in static conditions at 26°C, 14:10 light/dark cycle with 75 rpm horizontal agitation (Edmund Buehler, Germany). Four replicates were set up for each time point. At this concentration, the delayed hatching was also observed in all embryos of the OBI-UFZ lineage.

After exposure, embryos were dechorionated, washed twice with Milli-Q water, and transferred to 2 mL FastPrep tubes containing 0.75 mm glass beads. Excess water was removed. The tubes were snap-frozen in liquid nitrogen and stored at -20 °C until extraction and analysis. Samples of the exposure solution (300 µL) were also taken at the beginning and the end of exposure times, then stored at -20 °C.

For matrix-assisted laser desorption/ionization-MS imaging (MALDI-MSi) analysis, embryos exposed for 72 h and 96 h were washed twice and deposited in cryomolds containing Neg-50™ embedding medium (Thermo Scientific, Germany), frozen on dry ice, wrapped in aluminum foil, and stored at -70 °C.

2.4.2. Sample preparation and chemical measurements by UPLC-QToF-MS measurements

For measurements of the internal concentrations, the embryos in FastPrep tubes were extracted with 500 µL acetonitrile, homogenized in a FastPrep homogenizer (MP Biomedicals, 30 s, 6.5 m/s), placed in an ultrasonic bath for 15 min, and centrifuged for 15 min at 13000 rpm. 175 µL of the supernatant were then transferred to 1,5 mL glass vials filled with 175 µL Milli-Q water and stored at -20°C until analysis.

Both the aliquots taken from the exposure solutions and the embryo extract were analyzed by UPLC-QToF-MS with detailed instrumentation and procedures as described by Halbach et al. (2020). Moxidectin was detected as sodium adduct (m/z=662.367) at a retention time of 13.02 min. Moxidectin was serially diluted in a solution of water and acetonitrile (50:50) for calibration ranging from 0.78 to 100 ng/mL. Unfortunately, the dilution factor chosen for T0 aliquotes (DF = 10) did not allow for detection of moxidectin in those samples, therefore, it is assumed that the concentration is similar to the one measured after 24, 48, 72, and 96 h of exposure which were measured undiluted.

For recovery experiments, nine pooled embryos (≈ 100 hpf) from controls were extracted with 500 µL acetonitrile spiked with 50 ng/mL of moxidectin, in triplicates. Samples were measured with LC-MS/MS as well, using the same procedures and parameters described above. The recovery was 40.8%, and the internal concentrations in embryos were corrected for the results of the recovery experiments.

2.4.3. Slide Preparation for MALDI-MSi

Embryos in embedding medium were transferred into a -17°C cryotome chamber (Cryo-Star HM 560, Microm International, Walldorf, Germany) 24 h prior to sectioning. Sections (12 µm thick) were cut and subsequently deposited onto ITO-coated glass slides for MALDI (Bruker, Bremen, Germany). The slides were then dried in a desiccator (300 mbar) for 30 min before teaching marks were drawn around the sections, in the form of white marker crosses. Positive controls were produced by depositing one drop of a 10 ppm moxidectin solution in ethanol on negative control sections. After drying, slides were weighed to account for the amount of matrix to be deposited on them, as well as scanned for imaging (OpticLab H850, plustek, Ahrensfelde, Germany). Slides were then stored in the desiccator until spraying, which was carried out with an automatic sprayer (Image Prep, Bruker Daltonik, Bremen, Germany). Standard settings were used for HCCA application. The slides with embryo sections were sprayed with 7 µg/L HCCA in methanol/water/trifluoroacetic acid 49.9/49.9/0.2. MALDI-MSi measurements were carried out within 24 h after spraying.

2.4.5. MALDI-MSi

The slides were loaded into an FT-ICR MS (Solarix XR 12T, Bruker Daltonics, Germany) coupled to a MALDI ion source equipped with a 1 kHz Laser of 355 nm (Smartbeam II, Bruker Daltonics, Germany). The software ftmsControl (Bruker Daltonics, Germany) was used for setting optimal parameters and for measurements, while measurement areas were set with the FlexImaging software (Bruker Daltonics,

Germany). The following MALDI settings were applied: 14% laser power, 100 laser shots, 2000 Hz frequency, small laser focus, 50 µM raster width. Moxidectin was measured as potassium adduct (calculated *m/z* = 678.340) in positive mode and an isolation window of *m/z* = 662 (±30) was applied. MALDI data and scanned images were imported into SCiLS Lab 2020a (SCiLS GmbH, Bremen, Germany) for data analysis. Total ion count normalization, weak denoising and hotspot removal were applied.

2.5. Statistical analysis

The effect concentrations (EC_{50} and EC_{10}) were obtained with Probit analysis (Finney 1952). The results for biomarkers, heartbeat, and spontaneous movements were analyzed using the software GraphPad Prism 8 and are represented as mean ± standard deviation. The data were tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene's tests. Data fulfilling these requirements were analyzed by one-way ANOVA and *post-hoc* Turkey's test ($p < 0.05$).

The data retrieved from the UPLC-QToF-MS were analyzed using OriginPro statistical software, considering the volume (nL) of individual embryos at different developmental stages as previously described by (Halbach et al. 2020). The values were used to calculate the bioconcentration factor (BCF) as the internal concentrations divided by the concentration measured in the exposure solution (Wang 2016). The variations in internal concentrations, as well as concentrations in exposure solutions are expressed as mean ± standard deviation.

3. Results

3.1. Acute toxicity

At the highest nominal concentration tested (20 mg/L), 100% of the embryos failed to hatch but no mortality was observed (Table 1). Successive exposure assays revealed a delay in hatching that appeared in a concentration-dependent manner (EC_{50} = 18.98 µg/L, Figure 1).

3.2. Enzymatic activity

Changes in enzyme activity levels were observed at concentrations under the EC_{50} (1.25, 2.5, and 5 µg/L). The enzymes GST, LDH, and AChE (Figure 2, A-C) had significantly higher activities than the control in all treatments, while CAT (Figure 2, D) only showed an increase in its activity in the median concentration (2.5 µg/L). A trend of increased activity peaking at 2.5 µg/L was observed for all enzymes, except for LDH, which peaked already at the lowest concentration (1.25 µg/L) and then decreased.

At these concentrations, the number of spontaneous movements at 24 hpf and the heart rate at 48 hpf were measured, but no differences between treated groups and the control were observed (Figure 2, E-F).

3.3. Chemical analysis in embryos and exposure solutions

The measured external concentration was strongly below the nominal concentration (Figure 3, A). This may be explained with the known rapid photolysis of

moxidectin and possible sorption to equipment due to the high lipophilicity (log *P* = 6; Prichard et al., 2012).

The concentrations in whole embryos averaged 94.91 µM (± 74.33), in a strong contrast to the 94.82 x 10^{-4} µM (± 92.62 x 10^{-4}) found in exposure solutions (Figure 3). At the exposure concentration, 100% of the embryos did not hatch. These values yielded a very high BCF at the end of the exposure time based on the measured external concentration (1.11x10^{4}, 96 h). However, the concentration of moxidectin detected in the exposure media was far below the intended nominal concentration of 640 µg/L (1.0003 µM). Considering the intended concentration yields a much lower BCF (151.95, 96 h), which still indicates a strong enrichment of the embryos. No transformation products were found in the external solution or the embryo extracts.

The strong deviation between the concentration in the exposure medium and the embryo points towards a stronger stability of moxidectin in the embryos. Nevertheless, measurements of several time points within the first 24h might be necessary to better elucidate the dynamics of moxidectin in the water, and how it compares to the uptake by the embryo.

3.4. Spatial distribution

MALDI-MSi measurements showed an accumulation of moxidection in the head and eye regions compared to the rest of the embryo in embryos at 72 and 96 hpf (Figure 4).

4. Discussion

Despite the absence of lethal endpoints as defined by the OECD (OECD 2013), moxidectin caused a marked decrease in hatching rates at 96 h. The decline in hatching rates is an outcome of particular environmental relevance, as it can translate into decreased hatching viability and altered population dynamics (Parra et al. 2005; Navis et al. 2013). Additionally, a relatively low derived EC_{50} value (18.98 µg/L; ≈ 0.03 µM) indicates a high acute toxicity to zebrafish embryos. However, it can be difficult to determine how close this is to environmentally relevant concentrations because studies quantifying moxidectin in environmental samples are scarce. Zrnčić et al. (2014) detected moxidectin in a sample from a Spanish river at a concentration of 1.84 ng/L. However, the study collected water samples mostly close to pig farms, whereas the use of macrocyclic lactones tends offer more environmental risks in extensive cattle farming (Di Nica et al. 2015), and 32.2% to 52% of moxidectin administered to cattle and sheep is eliminated in the feces as the unchanged parent compound (Afzal et al. 2002; Zulalian et al. 2002). Thus, the level of moxidectin contamination in a given location depends on the predominance and composition of herds, as well as the volume of use relative to other antiparasitics (Kools et al. 2008; Di Nica et al. 2015).

Another aspect of the environmental relevance of moxidectin is its high potential to enrich in zebrafish embryos. Similar to ivermectin, moxidectin is a highly hydrophobic compound (log P = 6, Prichard et al. (2012) that binds to organic matter, allowing for high intake by living organisms through direct contact with the chemical or by consumption of contaminated organic matter (Mesa et al. 2018, 2020). In fact, moxidectin is persistent in dung and soil while being quickly degraded in water by photolysis (with a half-life of 6.8 h) (Fort Dodge Animal Health 1997; Hentz et al. 2019). Our results

showed that, despite its comparable low concentration in the medium, moxidectin is quickly taken up by the embryo in high amounts. As the concentration in the embryo did not drastically change over the 96 h of exposure, organic constituents in the embryo might also increase the stability of moxidectin in the embryo compared to the exposure solution. This also points out the need to consider toxicity of compounds rapidly degraded in water bodies to aquatic organisms. This result may be particularly relevant in chronic or tandem exposure situations, as this rapid uptake facilitates the persistence of the compound within the organism.

When embryos were exposed to concentrations of moxidectin that were too low to prevent hatching, all treatments increased AChE activity, peaking at the nominal concentration of 2.5 µg/L and subsequently decreasing. This trend was observed for the oxidative stress-related enzymes measured (CAT, LDH, and GST) as well, with catalase only reaching statistically higher activity levels at 2.5 µg/L (Figure 2). The data thus suggest deployment of compensatory mechanisms at the cell and molecular levels, followed by their exhaustion. This is in line with a recent theoretical framework proposing that fish will adopt a "conservation" metabolic strategy when faced with an overwhelming intensity and/or several stressors (Sokolova et al. 2012; Sokolova 2013; Petitjean et al. 2019). Considering xenobiotics as a type of environmental stressor, moxidectin may elicit a compensation strategy in concentrations below 2.5 µg/L in zebrafish embryos, but its effects overwhelm antioxidant systems from that point onwards. Moreover, all biochemical parameters showed statistically significant responses at the lowest concentrations tested, while spontaneous movements and heartbeats per minute were not sensitive measures of moxidectin toxicity. Since the latter are secondary-level stress responses (Schreck and Tort 2016), their refractory state further corroborates the adoption of a conservation strategy by the embryos.

The increase in GST activity is especially important because this enzyme participates directly in xenobiotic metabolism. While CAT and LDH are part of unspecific first-line antioxidant systems, GST facilitates the excretion of toxicants by conjugating them with reduced glutathione (Domingues et al. 2016), so our results show that moxidectin elicits oxidative stress responses involving both antioxidant and detoxification systems. On the other hand, LDH is widely distributed in the organism and is involved in anaerobic metabolism, so its activity can be differentially impacted in several body parts of fish, indicating losses in tissue integrity or low oxygen availability (Arya et al. 2010; da Silva Santos et al. 2018; Dar et al. 2020). Indeed, the increase in LDH could reflect a cytotoxic fallout, as evidenced by neurotoxic signals that may stem from skeletal muscle damage. In the lowest concentrations tested, moxidectin did not produce a significant difference in heart beats per minute or spontaneous movements, but LDH activity was higher in all treated groups, indicating that the drug could elicit myotoxic effects via upregulation of anaerobic metabolism (Tonomura et al. 2009; Arya et al. 2010). To further elucidate these questions, histopathological examinations may provide a qualitative and spatial understanding of moxidectin damage on tissues, and larval motility studies could be more sensitive to moxidectin toxicity at lower concentrations.

The increased AChE activity in all treatment groups, together with the decreased hatching rate, demonstrates that moxidectin is neuroactive in zebrafish, which is expected for most MLs because of their intrinsically neurotoxic mode of action (Lumaret et al. 2012). MLs have been shown to affect AChE activity differently: while eprinomectin inhibits brain AChE in jundiá fish (*Rhamdia quelen*), ivermectin inhibits it in zebrafish embryos but not in adults (Oliveira et al. 2016; Serafini et al. 2019). Generally, neurotoxicity induced by pesticides is associated with AChE inhibition (Minovski et al. 2019), but an increased presence or activity of AChE at the synaptic cleft results in higher

degradation of acetylcholine, ultimately reducing the activity of this ubiquitous neurotransmitter (Teodorak et al. 2015). This could play a role in the delayed hatching by inducing a depression in neuromuscular function and subsequently impacting the embryo's physical ability to hatch.

The MALDI-MSi analysis supports this pattern of neurotoxicity by detecting moxidectin in the eye and head regions of the embryo. This accumulation of a neurotoxicant in the head region associated with changes in AChE has also been observed in zebrafish embryos exposed to the neurotoxic pesticide naled (Halbach et al. 2019). Moreover, several neuroactive drugs have been shown to impact hatching either by decreasing hatching success at 96 h or by delaying its normal occurrence between 48 and 72 h (Cavalcante et al. 2017; de Farias et al. 2019; Massei et al. 2019). In a study with four antidepressants in isolation and in mixtures, (Nowakowska et al. 2020) demonstrated that fluoxetine and sertraline decreased hatching rates at 96 h, while all compounds and mixtures accumulated in zebrafish embryos.

The fact that moxidectin was detected in the head of embryos is also noteworthy because it indicates that the chemical bypasses the blood-brain barrier (BBB) in zebrafish larvae, either by crossing it or by preventing its proper development. In mammals, MLs do not readily cross the BBB (Kircik et al. 2016). However, ivermectin has been shown to reach the central nervous system of adult Atlantic salmon (Høy et al. 1990), and fish appear to be more sensitive to ML neurotoxicity (Lumaret et al. 2012). Sensitivity to intoxication by MLs in mammals is attributed to deficient P-glycoprotein activity – an efflux transporter encoded by multi-drug resistance genes (*mdr*) that non-enzymatically prevents MLs from crossing the BBB (Dowling 2006; Geyer et al. 2009). The function and structure of the BBB in zebrafish are conserved, developing between 3 and 10 dpf, but the spatio-temporal aspects of its development remain largely unknown (Kim et al.

2017; O'Brown et al. 2018). Therefore, the deposits of moxidectin in regions that correspond to the brain in larvae (72 and 96 hpf) could be due to the chemical reaching precursor regions before a functional BBB is fully present, or disrupting its development altogether.

The deposition of moxidectin in the eyes could be a result of accumulation in the neuronal components of sight, since zebrafish eyes are structurally conserved, but concentrate half of the neurons in early larvae (7-8 dpf), accounting for nearly a quarter of their total body volume (Zimmermann et al. 2018). In fact, the time of hatching for zebrafish embryos aligns with variations in light cycles, with embryos exposed to complete darkness developing more slowly and hatching later (Villamizar et al. 2013). Additionally, ivermectin intoxication in several species manifests as a combination of central nervous system clinical signs and retinal oedema (Teare and Bush 1983; Muhammad et al. 2004; Kenny et al. 2008; Epstein and Hollingsworth 2013), so moxidectin may also be able to cause ophthalmic damage in zebrafish.

5. Conclusions

Our results show that the effects of moxidectin on zebrafish embryos follow a pattern of neurotoxicity and oxidative stress, manifesting as a decline in hatching rates as late as 96 h, as well as enzymatic alterations. These biochemical alterations continued in concentrations below 5 µg/L (when no more macroscopic effects were observed), but neither the spontaneous tail movements nor the heart rate were sensitive enough to reflect the difference found in AChE, CAT, GST or LDH activity, indicating that behavioral assays may be necessary to provide a reliable picture of moxidectin toxicity in lower concentrations.

Moreover, we integrated phenotypic and enzymatic activity assays with internal concentration and distribution analysis of moxidectin in zebrafish embryos. We demonstrated that distribution of chemicals within the embryo is important to consider when investigating internal concentration and that the spatial distribution can support the understanding of toxicity to non-target organisms. Moxidectin is highly enriched in the embryo and was detected in the head and eye regions, which are rich in nervous tissue. The results thus corroborate the need to prioritize moxidectin as pointed out by previous studies to further elucidate the risks posed by this drug to non-target organisms and to subsidize mitigation strategies accordingly. To this end, a better understanding of the ecotoxicological profile and environmental fate of moxidectin is urgently needed.

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Figure captions

Figure 1. Zebrafish embryos at 96 hpf. Negative controls (A) hatched between 48 and 72 hpf, while moxidectin-exposed embryos failed to hatch by 96 hpf (B, T 96 h, 10 µg/L). The effect was documented as percentage (%) of hatched embryos at 96 h (C).

Figure 2. Enzymatic activity of acetylcholinesterase (AChE, A), lactate dehydrogenase (LDH, B), glutathione S-transferase (GST, C) and catalase (CAT, D), of zebrafish embryos exposed to moxidectin for 96 h. Additionally, spontaneous movements (24 hpf ± 1 h) and heart rate (24 hpf ± 1 h, F) were measured. The data were analyzed by one-way ANOVA and *post-hoc* Turkey's test. Results are expressed as mean ± standard deviation, with different letters indicating significant difference from the control ?? ($p < 0.05$).

Figure 3. Moxidectin concentrations (µM) in embryonic bodies as determined by UPLC-QToF-MS (n = 3, detected as an Na-adduct, *m/z* = 662.367). Values are expressed as mean ± standard deviation.

Figure 4. Spatial distribution of moxidectin in zebrafish embryo as determined by MALDI-MSi. Color-scale pixels represent the peak intensity found (detected as an K-adduct, m/z = 678.340). Results are depicted as MALDI-MSi results (left) and original

scanned images without MALDI-MSi markings (right). Arrows indicate the eyes. NC = negative control. PC = positive control.

Table 1. Apical effects of moxidectin on zebrafish embryos after 96 h of exposure.

Lethal and non-lethal endpoints	Toxicity metrics (96 h exposure)
Mortality	LC_{50} > 20 mg/L
(embryo coagulation, non-detachment of the tail bud, lack of somite formation, lack of heartbeat)	
Delayed hatching[*]	EC_{50} 18.98 µg/L
	(9.943 – 36.217)[**]
	EC_{10} 1.81 µg/L
	(0.948 – 3.454)[**]

[*]Only effect observed at the end of exposure to the maximum tested concentration of 20 mg/L.

[**]Lower and upper limits in µg/L.

Figure 1.

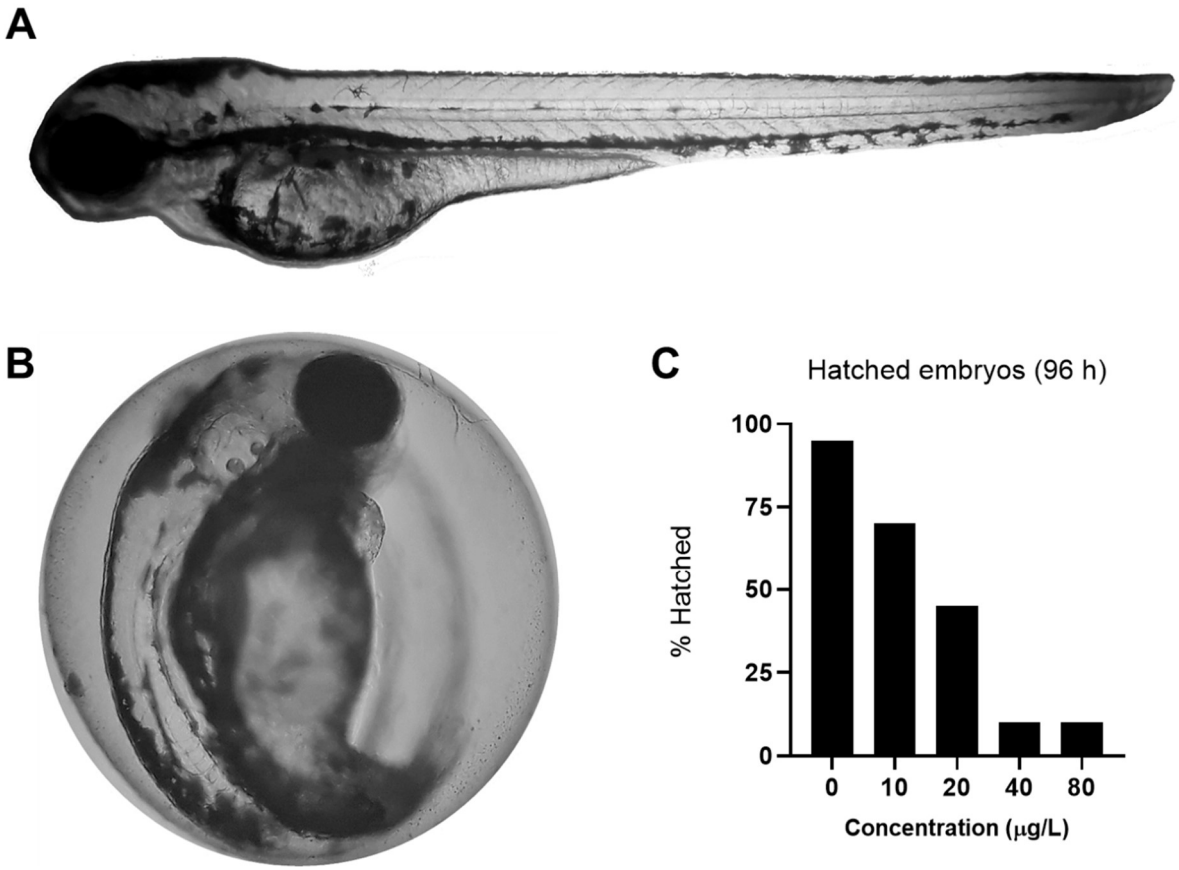

A
B
C
Hatched embryos (96 h)
% Hatched
100
75
50
25
0
0
10
20
40
80
Concentration (μg/L)

Figure 2.

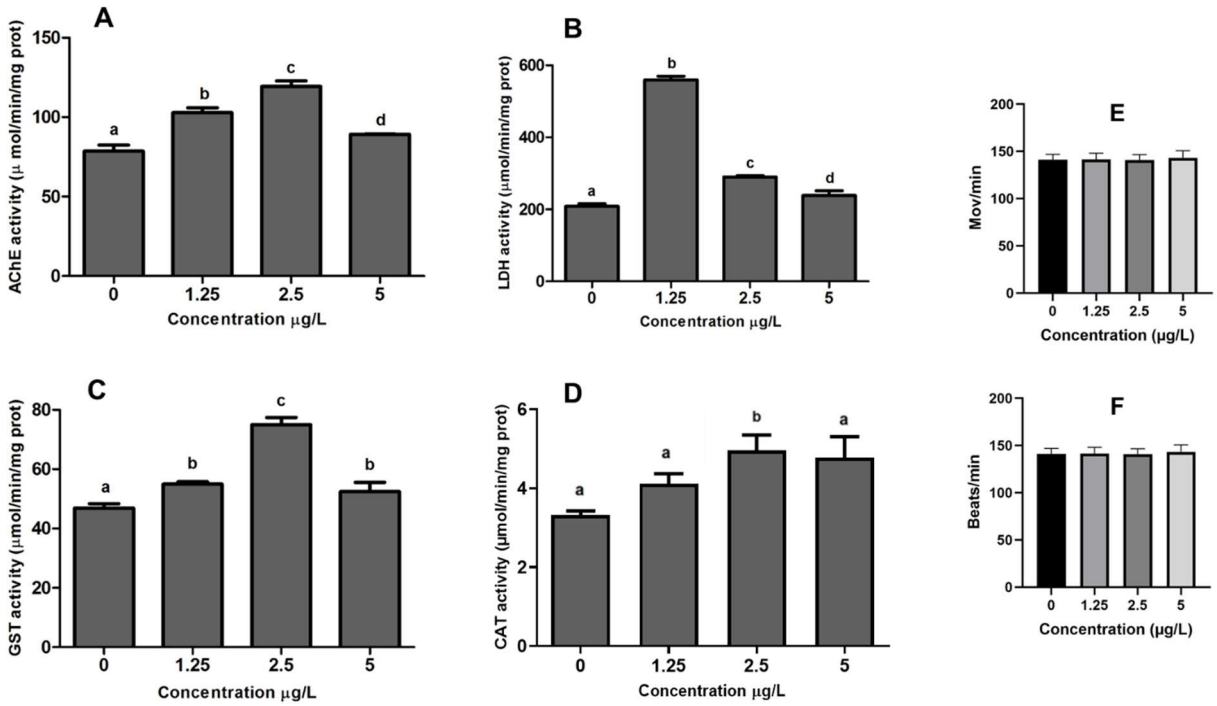

A
AChE activity (μ mol/min/mg prot)
150
100
50
0
a
b
c
d
0
1.25
2.5
5
Concentration μg/L
B
LDH activity (μmol/min/mg prot)
600
400
200
0
a
b
c
d
0
1.25
2.5
5
Concentration μg/L
E
Mov/min
200
150
100
50
0
0
1.25
2.5
5
Concentration (μg/L)
C
GST activity (μmol/min/mg prot)
80
60
40
20
0
a
b
c
b
0
1.25
2.5
5
Concentration μg/L
D
CAT activity (μmol/min/mg prot)
6
4
2
0
a
a
b
a
0
1.25
2.5
5
Concentration μg/L
F
Beats/min
200
150
100
50
0
0
1.25
2.5
5
Concentration (μg/L)

Figure 3.

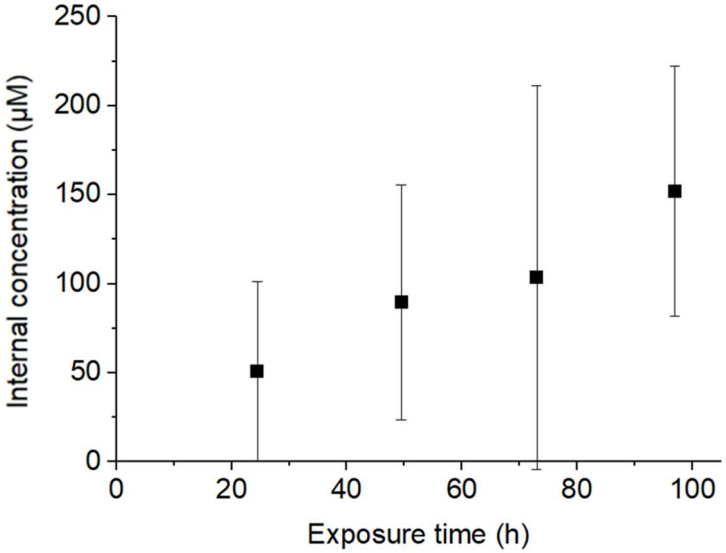
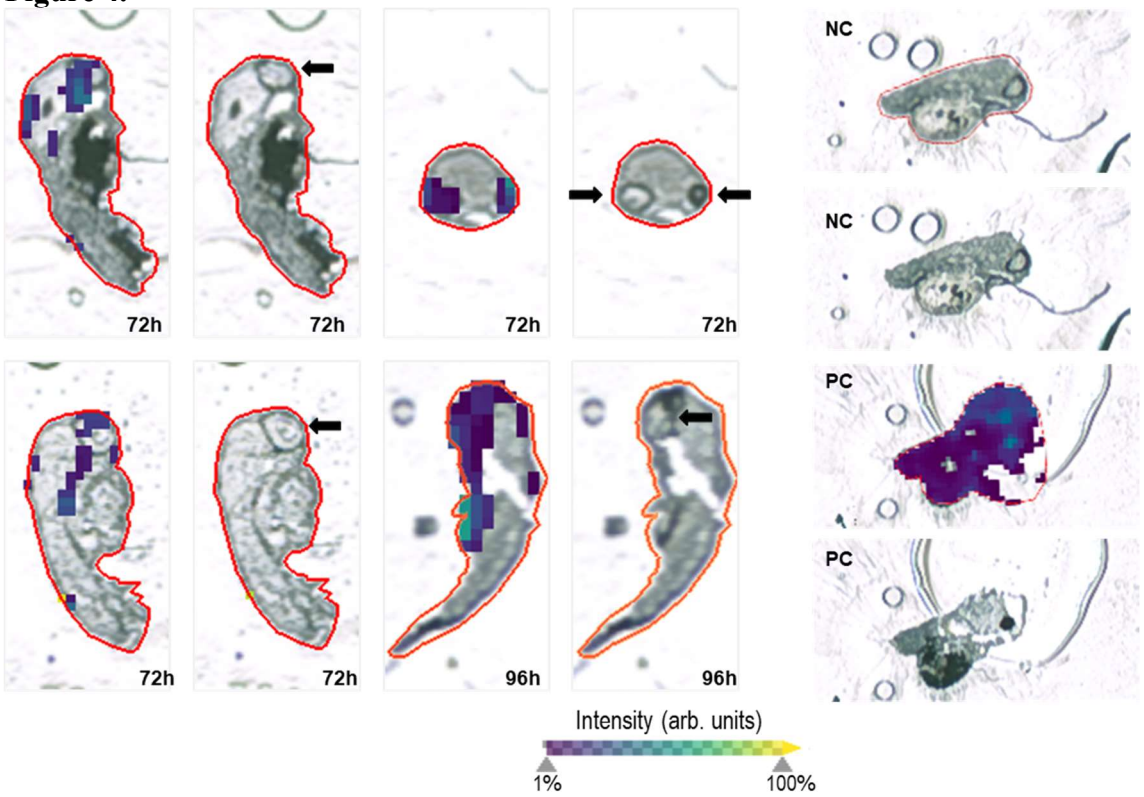

Internal concentration (μM)
250
200
150
100
50
0
0
20
40
60
80
100
Exposure time (h)

Figure 4.


72h
72h
72h
72h
NC
NC
72h
72h
96h
96h
PC
PC
Intensity (arb. units)
1%
100%

CAPÍTULO III
Artigo de revisão sistemática para submissão futura

The emerging picture of veterinary antiparasitics in aquatic environments

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Abstract

Veterinary antiparasitics are a diverse group of chemical compounds used worldwide for both treatment and prophylaxis of various herds with high economic and environmental impact. Together with their biocidal nature, this means that they may pose high environmental risks to aquatic wildlife. However, this group has not received much scientific attention in terms of studying their environmental risks, so we aimed to provide insights into the state of the environmental research on veterinary antiparasitics, with emphasis on toxicity to non-target organisms. We searched for relevant information on veterinary antiparasitics on PubMed, using three keyword combinations: (i) "veterinary antiparasitic" and "aquatic environment"; (ii) "veterinary parasiticide" and "aquatic environment"; and (iii) "veterinary antiparasitic", "aquatic" and "non-target". Our search yielded a total of 19 papers. For the toxicity studies, each individual organism and each individual compound within a paper was counted as one entry. This approach resulted in a diverse yield of chemical classes, test organisms, and methodological approaches. Prominently, we found a high frequency of entries for invertebrate taxa (56 entries; 60.71%). Additionally, macrocyclic lactones and benzimidazoles were the most important chemical groups, collectively accounting for 66.67% of entries. Moreover, our findings indicate a trend of toxicity towards invertebrates, but most studies were carried out in laboratory settings using standardized protocols. Based on our results, we posit that veterinary antiparasitics warrant coordinated action to understand their environmental risks.

Keywords: aquatic toxicity, veterinary parasiticides, environmental risk assessment, emerging contaminants, agriculture, pollution.

1. Introduction

As concerns over medicines in the environment grew, Boxall et al. (2003) discussed the role of veterinary pharmaceuticals and their potentially novel routes of contamination and the major gaps to be filled in order to identify and address their environmental risks. Veterinary pharmaceuticals reach aquatic environments through routes that depend on the production system in which they are used, as well as the host being treated and the route of administration. Examples of higher-risk activities are net pan enclosures at fish farming facilities and treatment of grass-fed cattle near water bodies, which are made greater when applied to large-scale herds (Kim et al. 2008; Di Nica et al. 2015).

Within the broader category of veterinary pharmaceuticals, antiparasitics (also referred to as parasiticides) are drugs used to control parasites such as helminths and ticks, and they present a set of features that may characterize their environmental relevance. They reach the environment through novel routes described for veterinary pharmaceuticals at large, but in large volumes and causing detrimental effects that comparable to those of antibiotics (Di Nica et al., 2015; Kools et al., 2008). The agricultural landscape is of particular importance for the environmental risks posed by large-scale use of veterinary antiparasitics (Di Nica et al., 2015; Kools et al., 2008). Plagues such as ticks, fleas, flies and helminths are a continuous threat of financial loss in livestock production systems, so they must be closely controlled, leading to prophylactic and continuous use of parasiticides (Kornele et al. 2014; French 2018). In the absence of specific limiting regulations, these drugs may be extensively and indiscriminately used, exerting high levels of xenobiotic-related stress to aquatic organisms in nearby ecosystems (Vieira et al. 2019).

Parasiticides are often not fully metabolized and are excreted via urine and feces by treated animals. One of the oldest and most widely used parasiticide, ivermectin, is only partly metabolized by the liver, then excreted mostly through the feces – between 40 and nearly 80% (Halley et al. 1989; Chiu et al. 1990; Lifschitz et al. 2000). Many antiparasitics, such as carbamates and pyrethroids, are also available in formulations for external use (e.g. pastes, sprays, aspersion, and baths) to treat ectoparasites such as fleas, ticks and flies. In these cases, metabolization is irrelevant, as the compounds may reach nearby ecosystems via wash-off (Boxall et al. 2003). For aquatic environments, common routes of pollution include direct deposition by animals reared in pasture, lixiviation to nearby bodies of water, and incorrect waste disposal (Kim et al., 2008). Drugs used in aquaculture such as emamectin benzoate and teflubenzuron are particularly concerning in the area around facilities, possibly reaching further (Bloodworth et al. 2019).

Once they reach aquatic ecosystems through such routes, these drugs exert a broad set of ecotoxicological effects. In an extensive review that aimed to assess the non-target effects of macrocyclic lactones in aquatic and terrestrial environments, Lumaret et al. (2012) discussed a tendency for these compounds to be more toxic to aquatic invertebrates, especially during early life stages. In addition, Carlsson et al. (2013) sought to assess the adverse effects of 15 veterinary pharmacists (10 antiparasitic and 5 antibiotics) on zebrafish embryos and observed several endpoints such as mortality, malformations and other sublethal responses, suggesting that this high toxicity may extend to early life stages of fish. This may be a product of the evolutionary relationships bootstrapping the parasite, the host and the non-target species (Brady et al. 2017), as many of these chemicals disrupt conserved structures that are present in target parasites (mainly arthropods and helminths), as well as in non-target organisms. Examples are

acetylcholinesterase inhibitors such as organophosphates, and beta-tubulin inhibitors like benzimidazoles (Anadón et al. 2009; Akre 2016; Zhang et al. 2020).

Additionally, indicators such as mode of action and market size might help understand the environmental risks posed by veterinary parasiticides. For example, macrocyclic lactones, pyrethroids and pyrethrins are examples of chemicals used worldwide and in high volumes for cattle production (Di Nica et al., 2015; Kools et al., 2008). These compounds disrupt the activities of ion channels that are conserved in invertebrates, leading to neurotoxic effects in several non-target species (Lumaret et al. 2012). Considering the large number of animals used in major cattle-producing countries, veterinary antiparasitics as a whole warrant attention. In 2018, flea and tick medications accounted for 29.4% of the U.S. animal health market (Pham and Donovan, 2018), while antiparasitics have been the most important therapeutic class in the Brazilian animal health market since 2015, having surpassed antibiotics in 2014 (SINDAN, 2019). This is in line with the arguments used by Wardhaugh (2004) to draw attention to parasiticides used in livestock production, warning of the need for usage information and the potential risks to non-target organisms in dung and pasture. Since then, other such calls for research on the matter have appeared in the literature (Loeb 2018; Powell et al. 2018). Despite this, studies on many antiparasitic drugs are still scarce, especially those considering their effects on aquatic organisms.

This study aims to provide a preliminary picture of the role of veterinary antiparasitics as environmental toxicants in aquatic environments. We use a systematic review as a steppingstone to examine veterinary antiparasitics as a separate chemical entity in terms of their risks to non-target aquatic wildlife.

2. Material and methods

To guide the search, selection, and analysis of scientific papers, we used the following research question as a guide: "what are the risks imposed to non-target aquatic organisms by large-scale use of veterinary antiparasitics?". This question helped to delineate the inclusion and exclusion criteria, as well as the information systematically retrieved. It also helped to broaden the scope of studies included, considering not only the toxicity of the chemicals, but also other research that could be useful to answer the guiding question.

Using PubMed (https://pubmed.ncbi.nlm.nih.gov/), we gathered the scientific articles retrieved between August 2019 and June 2020 by the following keyword combinations: (i) "veterinary antiparasitic" and "aquatic environment"; (ii) "veterinary parasiticide" and "aquatic environment"; and (iii) "veterinary antiparasitic", "aquatic" and "non-target". The keywords combinations were an attempt to maximize the number of results, considering that antiparasitics may be described as parasiticides as well. With the same purpose, we did not exclude based on publication date. The articles retrieved were retrieved between December 2019 and July 2020, then curated by the following inclusion and exclusion criteria:

i. Inclusion criteria: studies on compounds used in treatment of parasites in animals, toxicity to aquatic organisms, risk assessment contributions, studies on environmental samples (e.g. biological tissue, sediment, water), peer-reviewed literature.
ii. Exclusion criteria: studies on other pharmaceuticals groups (e.g. antibiotics), efficacy studies, clinical cases, analysis in products for human consumption, review papers, studies in languages other than English, non-aquatic organisms.

The included papers were then scanned for relevant information, which included their objective, chemicals and organisms studied, exposure conditions, effect observed, toxicity metrics (LC_{50}, EC_{50}, NOEC), type of samples taken in environmental fate studies, detection method.

3. Results

The combinations of keywords yielded a total of 65 unique results, of which only 20 were left once the inclusion/exclusion criteria were applied. These articles were arranged in three categories according to their objectives and information provided, namely: toxicity testing (table 1), environmental fate (table 2) and other (table 3). This third category was necessary to accommodate relevant information that did not fit into toxicity or environmental fate studies. It comprised one paper about the uptake and depuration kinetics of selected veterinary pharmaceuticals in blue mussels (Brooks et al. 2019), and two related to environmental risk assessments (Kools et al. 2008; Liebig et al. 2010).

To quantitatively record the data retrieved from toxicity studies, we used an entry system in which one entry equals the study of one chemical in a single organism. In this manner, one paper may correspond to one or more entries, depending on how many chemicals and how many organisms it investigated.

Chemical compounds

A total of 12 articles on toxicity to non-target organisms were included in this study (Table 1). To provide a more concise analysis, we registered one entry per compound studied in each paper, then we categorized them by chemical group (Figure 1). Macrocyclic lactones (MLs) and benzimidazoles (BZs) were the most studied chemical groups. Taken together, these groups accounted for 66.67% (12 entries) of the individual entries in toxicity papers. As for specific compounds, the most investigated of the MLs

was ivermectin (16.67% of entries), while fenbendazole (16.67% of entries) was the most studied BZ.

In the category of environmental fate studies, we had a total count of five articles and seven entries, with MLs appearing in 71.43% of entries and BZs in 28.57% (Table 4). Only two studies investigated more than one compound: one with fenbendazole and flubendazole (BZs), and one with emamectin benzoate (ML) and teflubenzuron, a benzoylurea. The studies used a range of analytical chemistry methods to study either the properties or the presence of the compounds in soil, sediment, water and/or fish muscles. Samples were obtained in their respective countries, namely: Greece, Scotland, Brazil, and Poland.

Non-target organisms

While the number of individual studies on toxicity to non-target organisms was limited (12 papers), only two of them were single-species investigations. Additionally, four articles included toxicity data on 10 organisms or more, which resulted in a relatively large number of entries for non-target organisms tested (56 entries in total). Invertebrates were the most studied group of organisms, accounting for 60.71% of the total number of entries (Figure 2). This category included all animals outside the phylum Chordata. Among them, crustaceans were the most predominant, with daphnids appearing most frequently (14.29%) and *Daphnia magna* occupying the position of most studied individual species (10.71%). *D. magna* also proved to be a sensitive test organism, being the most sensitive organism to five of the compounds, as well as yielding some of the lowest mean effect concentration (EC_{50}) in the studies included (Tables 4 and 5).

Because of the diversity of test organisms in the studies included, we divided them in rough biological groups, rather than taxonomically sound categories (Table 4). This

diverse pool of test organisms also brought an assortment of endpoints that ranged from the individual level to the population level (from liver gene expression to community abundance). The level of identification of the organisms also varied, with some studies distinguishing between individual species in each genus, while others only provided order- and phylum-level information on the studied organisms (e.g. Nematoda, Cladocera, Tardigrada). Whenever provided, we also comprehensively include metrics such as EC_{50} values to compare toxicity among biological groups, as described in Tables 4 and 5.

Discussion

Despite the small number of articles, the results provide a strong framework for discussion, since they encompass a highly diverse set of methods, test organisms, and chemical compounds. Prominently, the high number of test organisms (representing a few major biological groups) and the diversity of chemical classes allowed for discussions of taxa-specific toxicity.

Among the compounds included, the overrepresentation of macrocyclic lactones and benzimidazoles falls in line with the wide use of these major classes of pharmaceuticals. The ML ivermectin is a major parasiticide worldwide, used to treat various diseases such as worm infections in animals and river blindness in humans (Molento 2020). The benzimidazoles are also prominent drugs used worldwide to treat parasitic and fungi-related illnesses in humans and animals (Brauer et al. 2019; Porto et al. 2020).

Overall, the trend of higher toxicity to invertebrates reflects the fact that antiparasitics are designed to target this group. Many active ingredients in antiparasitics,

such as pyrethroids and organophosphates, are commonly found in insecticide and pesticide formulations (Akre 2016). Other active ingredients in veterinary antiparasitics include pyrethrins and carbamates (Anadón et al. 2009; Akre 2016), which illustrates how these pharmaceuticals intersect with pesticides in terms vulnerable non-target organisms. Most importantly, parasiticides, agricultural pesticides and insecticides are designed to maximize toxicity to specific invertebrate taxa (mostly arthropods and nematodes), and in many cases optimized for conserved structures to allow a broader spectrum of activity (Powell et al. 2018). As a result, it is unsurprising that cypermethrin (a pyrethroid used to control several arthropods such as aphids and flies) is highly toxic to *Amphiporeia virginiana*, and aquatic arthropod (Table 3). This "toxicity by design" could by why Kools et al. (2008) and Di Nica et al. (2015) found that antibiotics and parasiticides typically ranked high in their risk-based assessments of veterinary pharmaceuticals.

Accordingly, the retrieved EC_{50} data point to a trend of higher toxicity towards invertebrates, especially microcrustaceans such as *D. magna*, which was the most sensitive species to five out of the eight compounds (ivermectin, fenbendazole, trichlorfon, abamectin, and doramectin) tested in more than one species. In fact, the ubiquity of *D. magna* as a test organism, combined with its low EC_{50}s frequently placed it as the most sensitive species in multi-species studies (Tables 1 and 3). This trend is in line with previous calls for research on veterinary antiparasitics that were primarily concerned with declines in populations of insects and small riverine invertebrates (Powell et al. 2018).

We favored EC_{50} to compare toxicities among non-target organisms because substantially more articles disclosed EC_{50}s than LC_{50}s or NOECs. We also consider that EC_{50} values represent a more sensitive metric compared to LC_{50}, as many chemicals still produce detrimental effects that manifest at the population level without causing

mortality. Additionally, only specific effects are required by many standardized protocols, such as the OECD Test No. 211, which tests the reproductive output of *Daphnia magna* in response to chemical exposure (OECD, 2018). Despite not being lethal, this is an effect that leads to a reduction in population size. Prominent examples of this phenomenon of trickling up to detrimental populational outcomes are the disruption of developmental and neuronal processes. Benzimidazoles, for instance, are beta-tubulin inhibitors that interfere with cell division during early development of zebrafish, causing skeletal deformities and adversely impacting movement (Zhang et al. 2020), which ultimately reduces fitness. This type of sub-lethal drop in fitness can also be expected for chemicals with neurotoxic effects. Pyrethroids, pyrethrins, organophospates and carbamates are all classes of compounds that inhibit acetylcholisnestarase (AChE), which has been documented in several aquatic organisms, including fish, crustaceans, and clams (Toumi et al. 2016; Arora et al. 2017; Singh et al. 2018; Li et al. 2018). At the population level, neurotoxicity at large may lead to behavioral changes that increase predation or disrupt social behaviors (Sandoval-Herrera et al. 2019; Armstrong et al. 2019; Faria et al. 2020; Bedrossiantz et al. 2020). An aggravating factor for these classes is that they commonly appear as pour-on treatments, which provides a direct track for the compounds to reach nearby ecosystems unchanged (Loeb 2018).

However, mode of action and toxicity to the target species are not the only variables affecting the ultimate toxicity endpoints observed in a given species. Other factors such as time and length of exposure, chemical properties, experimental conditions, toxicokinetics and toxicodynamics, formation of derivates in the environment or by biotransformation, and specific biological peculiarities can cause disagreements in the effects between related species and chemicals. In a study with six benzimidazole-based antihelminthics, Oh et al. (2006) found various degrees of toxicity towards *D. magna.*

Given that the compounds are structurally similar, the authors argue that the differences can be explained by the octanol-water partition coefficient (K_{ow}) of the chemicals, which is a lipophilicity parameter. On the other side of the issue, Hong and Zha (2019) found different behavioral sensitivities between zebrafish and Chinese rare minnow (two teleost fish from the Cyprinidae family) exposed to imidacloprid and chlorpyrifos.

Finally, the span and endpoints investigated in individual toxicity investigations also varied greatly, but mostly focused on standardized acute toxicity assays, which is another aspect of the diverse test organisms. For example, the OECD protocol No. 201 (2011) requires a 72-hour exposure to assess growth inhibition in alga and cyanobacteria over several generations, while the guidelines for *D. magna* acute toxicity (OECD, 2004) require a 48-hour exposure. Longer living organisms such as zebrafish require a 96-hour exposure to assess acute toxicity towards embryos and 21 days to assess certain endocrine disturbances in sexually mature individuals (OECD, 2009; OECD, 2013). Additionally, one field study in our results relied on simulating operational schedules of azamethiphos and cypermethrin applications to salmon in net pan enclosures, taking two years to study the effects on various test organisms following three simulated applications, though the individual toxicity assays remained standardized (Ernst et al. 2001).

However, for the results to translate into ERA-relevant information, these hypotheses need to be investigated at several levels of biological organization, with tiered methodological approaches. The predominance of strictly laboratory-based tests in accordance with standardized guidelines (e.g. OECD, ISO), compared to a smaller number of field and microcosm studies is consistent with one of the two ERA papers in our pool (Liebig et al. 2010), which sook to establish a case-study, multi-tiered ERA for ivermectin. As noted by the authors, even though strictly laboratory-based assays provide useful data on non-target toxicity and chemical properties, more information and

standardized protocols at higher-tier levels are imperative. Additionally, Di Nica et al (2015) have documented the lack of chronic toxicity data as another potential source of hinderance.

Conclusions and perspectives

We provide a non-exhaustive, but comprehensive analysis of the toxicity of antiparasitic drugs to aquatic wildlife in the context of veterinary use. Macrocyclic lactones and benzimidazoles were the main classes studied, reflecting the well-established importance of these drugs in veterinary practice. The results also supported the speculated trend of toxicity towards invertebrates based on EC_{50} and NOEC values. Likewise, the high frequency of invertebrate entries (%) indicates a preference by the authors to use them as test organisms, demonstrating the importance of this biological group for the toxicity testing of antiparasitics.

Therefore, our results provided a basis for discussion covering the toxicity of antiparasitics of major importance to a large variety of test organisms. Given the importance of antiparasitic drugs in animal production systems worldwide, we posit that they warrant coordinated efforts to expand the literature about their environmental impacts. For this purpose, a range of methodological approaches may be necessary to inform prioritization and mitigation efforts. The data collected suggests that major priority should be given to quantification of compounds in environmental samples that can inform the significance of EC_{50} values. Higher-tier studies and chronic exposures may also provide realistic exposure scenarios that integrate variables related to both fate and toxicity. Additionally, toxicity assessments that include mechanistic and biochemical information (e.g. biomarker assays, bioaccumulation and biomagnification, trophic

transfer) may be valuable in refining the current information about the odds, routes, and impact of these chemicals.

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Figure 1. Frequency (%) of veterinary antiparasitics tested for toxicity to non-target organisms per chemical class (A); of biological groups used in toxicity assays with veterinary antiparasitics (B); and of invertebrates within those biological groups (C). In A, each entry represents a study of one compound within the chemical classe on a research article. In B and C, each entry represents a study of the effects of an individual chemical on a single test organism within a research article.

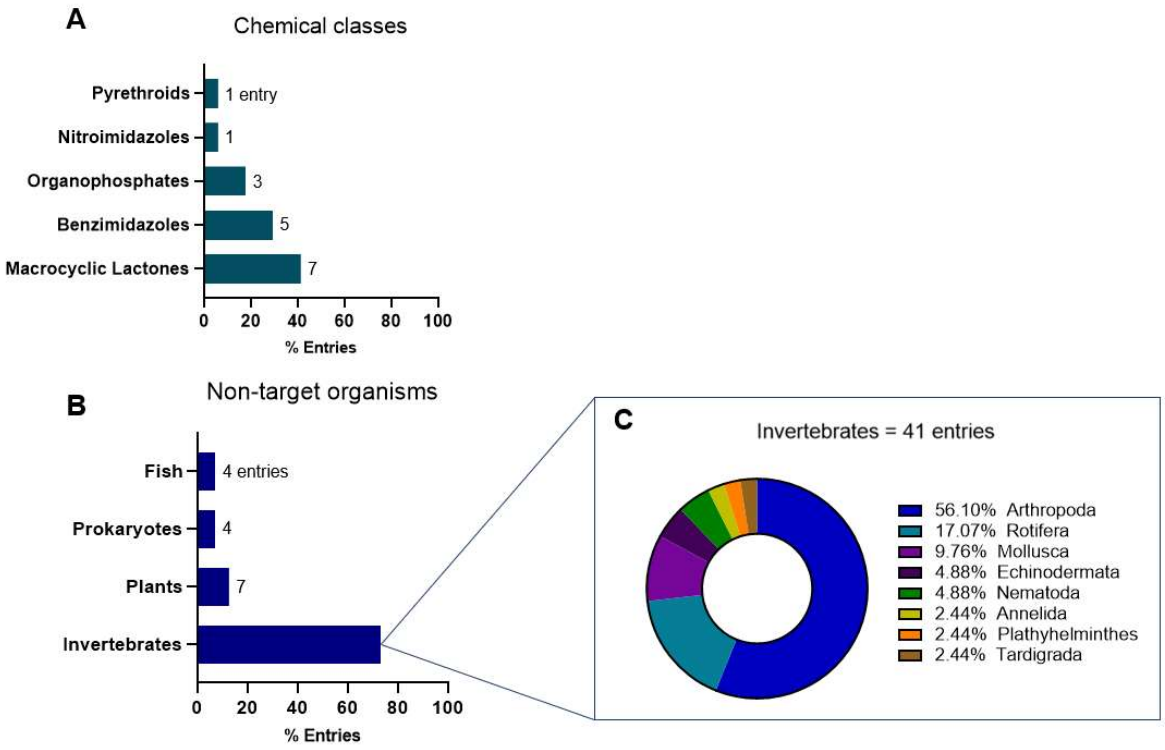

Table 1. Studies of the toxicity of various veterinary antiparasitics drugs to non-target organisms. Ref.: reference number.

Compound	Organism	Exposure conditions	Length of exposure	Effect studied	Observed effect (EC_{50} /LC_{50}) for antiparasitics	Reference	Ref.
Moxidectin	*Pomacea canaliculata*	Dung sparkled with moxidectin; water-sediment microcosm (OECD 218)	17 days	Survival and growth	Reduced survival from 250 µg/kg (17 days)	Mesa et al. 2018 (doi= 10.1007/s00244-018-0539-5)	1
Moxidectin	*Hyalella curvispina*	Dung sparkled with moxidectin; water-sediment microcosm (OECD 218)	17 days	Survival and growth	Mortality from 250 µg/kg (7 days)	Mesa et al. 2018 (doi= 10.1007/s00244-018-0539-5)	1
Moxidectin	*Ceriodaphnia dubia*	Dung sparkled with moxidectin; water-sediment microcosm (OECD 218)	17 days	Survival and growth	No statistically significant effects	Mesa et al. 2018 (doi= 10.1007/s00244-018-0539-5)	1
Eprinomectin	*Rainbow trout*	Laboratory settings	96 h	Tanscriptional changes and activity of antioxidant enzymes	Decreased SOD, CAT, 8-OHdG and GPx activities in the liver	Alak et al.2017 (doi=10.1016/j.fsi.2017.04.004)	2
Flubendazole	*Dugesia gonocephala*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h, immobility) = 21.9 µg/L (most sensitive)	BUNDSCHUH et al. 2016 (doi=10.1007/s00128-015-1656-8)	3
Flubendazole	*Caenorhabditis elegans*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(24h, immobility) = >1000 µg/l		3
Flubendazole	*Brachionus calyciflorus*	Laboratory settings (ASTM E1440)	Between 24 and 96 h	Immobility and toxicity	EC_{50}(24h, immobility) = >8000 µg/l		3
Flubendazole	*Tubifex tubifex*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h, immobility) = 22.1 µg/l		3
Flubendazole	*Radix ovata*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h, immobiliy) =1000 µg/l		3
Flubendazole	*Daphnia magna*	Laboratory settings (OECD 202, 2004)	Between 24 and 96 h	Immobility and toxicity	EC_{50}(48h) = 70.1 µg/l		3
Flubendazole	*Gammarus pulex*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 105.4 µg/l		3
Flubendazole	*Asellus aquaticus*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = >1000 µg/l		3
Flubendazole	*Amphinemura sulcicollis*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = >1000 µg/l		3
Fenbendazole	*Dugesia gonocephala*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 44.2 µg/l		3
Fenbendazole	*Caenorhabditis elegans*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(24h) = >1000 µg/l		3

Fenbendazole	*Brachionus calyciflorus*	Laboratory settings (ASTM E1440, 1998)	Between 24 and 96 h	Immobility and toxicity	EC_{50}(24h) =>8000 µg/l		3
Fenbendazole	*Tubifex tubifex*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 32.0 µg/l		3
Fenbendazole	*Radix ovata*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 1000 µg/l		3
Fenbendazole	*Daphnia magna*	Laboratory settings (OECD 202, 2004)	Between 24 and 96 h	Immobility and toxicity	EC_{50}(48h, Most sensitive organism) = 16.7 µg/l		3
Fenbendazole	*Gammarus pulex*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 146.4 µg/l		3
Fenbendazole	*Asellus aquaticus*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = >1000 µg/l		3
Fenbendazole	*Amphinemura sulcicollis*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = >1000µg/l		3
Ivermectin	*Dugesia gonocephala*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 675.2 µg/l		3
Ivermectin	*Caenorhabditis elegans*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(24h) = 17.5 µg/l		3
Ivermectin	*Brachionus calyciflorus*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(24h) = 1961 µg/L		3
Ivermectin	*Tubifex tubifex*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 1866 µg/l		3
Ivermectin	*Radix ovata*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(96h) = 17 µg/l		3
Ivermectin	*Daphnia magna*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50}(48h, highly toxic) = 0.59 µg/l		3
Ivermectin	*Gammarus pulex*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50} (96h, highly toxic) = 1.4 µg/l		3
Ivermectin	*Asellus aquaticus*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50} (96h) = 390.3 µg/l		3
Ivermectin	*Amphinemura sulcicollis*	Laboratory settings	Between 24 and 96 h	Immobility and toxicity	EC_{50} (96h) = 14.3 µg/l		3
Flubendazole	*Vibrio fischeri*	Laboratory settings (DIN 38412-L34, 1991)	30 min	luminescence inhibition	EC_{50} (48h, luminescence) = 300 µg/L	Wagil et al. 2015 (doi= 10.1007/s11356-014-3497-0)	4
Flubendazole	*Scenedesmus vacuolatus*	Laboratory settings (ISO 8692, 1989)	24 h	inhibition of algal reproduction	EC_{50} (48h, growth) = >1000 µg/L		4
Flubendazole	*Lemna minor*	Laboratory settings	3 days	growth inhibition	EC_{50} (48h, No effect on growth) = >1000 µg/L		4
Flubendazole	*Daphnia magna*	Laboratory settings (OECD 202, 2004)	48 h	immobilization	EC_{50}(48h, most sensitive organism) = 45 µg/L		4
Fenbendazole	*Vibrio fischeri*	Laboratory settings (DIN	30 min	luminescence inhibition	EC_{50}(48h, No effect on		4

		38412-L34, 1991)			luminescence) = 300 µg/L		
Fenbendazole	*Scenedesmus vacuolatus*	Laboratory settings (ISO 8692, 1989)	24 h	inhibition of algal reproduction	EC_{50} (48h, No effect on growth) = >1000 µg/L		4
Fenbendazole	*Lemna minor*	Laboratory settings	3 days	growth inhibition	EC_{50} (48h, No effect on growth) = >1000 µg/L		4
Fenbendazole	*Daphnia magna*	Laboratory settings (OECD 202, 2004)	48 h	immobilization	EC_{50}(48h, most sensitive organism) = 19 µg/L		4
Doramectin	*Vibrio fischeri*	Laboratory settings (DIN 38412-L34, 1991)	30 min	luminescence inhibition	EC_{50}= not reached	KOŁODZIEJSKA e t al. (doi=10.1016/j.chemosphere.2013.04.057)	5
Doramectin	*Scenedesmus vacuolatus*	Laboratory settings (ISO 8692, 1989)	24 h	inhibition of algal reproduction	EC_{50}= not reached		5
Doramectin	*Lemna minor*	Laboratory settings	3 days	growth inhibition	EC_{50}= not reached		5
Doramectin	*Daphnia magna*	Laboratory settings (OECD 202, 2004)	48 h	immobilization	EC_{50}(48h) = 0,0637 µg/L		5
							5
Trichlorfon	*Danio rerio (embryos and adults)*	Laboratory settings (OECD 203, 1992) (OECD 236, 2013)	(embryos)= 5 days (adults)= 96h	biomarkers (ChE, GST, CAT, LDH)	EC_{50}(48h, changes in biomarkers) = Embryos= 25400 µg/L| Adults= 28800 µg/L	Coelho e al. 2011 Doi= 10.1016/j.aquatox.2011.03.003	6
Trichlorfon	*Daphnia magna*	Laboratory settings (OECD 202, 2004)	48 h	immobilization + biomarkers	EC_{50}(48h, changes in biomarkers) = 0.29 µg/L		6
Trichlorfon	*Pseudokirchneriella subcapitata*	Laboratory settings (OECD 201, 2006)	96 h	growth inhibition	EC_{50}(96h, No effect on growth) = 274500 µg/L		6
Trichlorfon	*Chlorella vulgaris[MSM1]*	Laboratory settings (OECD 201, 2006)	96 h	growth inhibition	EC_{50}(not affected)		6
							6
Fenbendazole	*Chironomus riparius larvae*	Laboratory settings	96 h	Gene expression and biomarker activity	LC_{50} = 93.5 µg.L^{1}	Park et al. 2009 (doi=10.1016/j.chemosphere.2009.07.019)	7
							7
Ivermectin	*Ceriodaphnia sp*	Microcosm	42 days	Community abundance and acute toxicity	NOEC(6 days, most sensitive) = 0.03 µg/L	Boonstra et al. 2011 (doi= 10.1007/s00244-010-9526-1)	8
Ivermectin	*Chydorus sphaericus*	Microcosm	42 days	Community abundance and acute toxicity	NOEC (significant reductions) = 0.1 µg/L		8
Ivermectin	*Daphnia longispina*	Microcosm	42 days	Community abundance and acute toxicity	NOEC (significant reductions) = 0.3 µg/L		8

Ivermectin	Cyclopoida Nauplii	Microcosm	42 days	Community abundance and acute toxicity	NOEC (6-13 days, significant reductions) = 1 µg/L		8
Ivermectin	*Cephalodella sp.*	Microcosm	42 days	Community abundance and acute toxicity	NOEC (No effects) = 1 µg/L		8
Ivermectin	*Anuraeopsis fissa*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Lecane group lunaris*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Polyarthra remata*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Lecane group luna*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Brachionus quadridentatus*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Trichocerca group porcellus*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Keratella quadrat*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Ivermectin	*Synchaeta sp.*	Microcosm	42 days	Community abundance and acute toxicity	NOEC= 3 µg/L		8
Abamectin	*Vibrio fischeri*	Laboratory settings (ISO 11348–2, 1998)	30 min	luminescence	EC_{50} (30 min, low toxicity) = 690 µg/L	Tisler et al. 2006 (doi:10.1007/s10646-006-0085-1)	9
Abamectin	*Scenedesmus subspicatus*	Laboratory settings (ISO 8692, 1989)	72 h	Growth	EC_{50} (72 h) = 44 µg/L		9
Abamectin	*Daphnia magna*	Laboratory settings (OECD 211, 1998)	Acute= 48 h Chronic= 21days	immobility, mortality and reproduction	EC_{50}(48 h, decreased of mobility) = 0,25 µg/L (21 days NOEC)=0,0047 µg/L		9
Abamectin	*Danio rerio*	Laboratory settings (ISO 7346–1/ ISO 7346-2, 1996)	96 h	mortality and swimming activity	EC_{50}(24 h, decreased swimming) = 50.4 µg/L		9

Azamethiphos	*Vibrio fischeri*	Field study (dispersion from net pan enclosures)	15 min	luminescence	EC_{50} = 11000 µg/L	Ernst et al. 2001 (doi:10.1016/S0025-326X(00)00177-6)	10
Azamethiphos	*Amphiporeia virginiana*	Field study (dispersion from net pan enclosures)	48 h	mortality	Not tested		10
Azamethiphos	*Gammarus spp*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50} = <5 µg/L		10
Azamethiphos	*Eohaustorius estuarius*	Field study (dispersion from net pan enclosures)	48 h	mortality	LC_{50} = >20 µg/L		10
Azamethiphos	*Polydora cornuta*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50} =2310 µg/L		10
Azamethiphos	*Strongylocentrotus droebachiensis*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50} = >1000 µg/L		10
Azamethiphos	*Brachionus plicatilis*	Field study (dispersion from net pan enclosures)	24 h	mortality	---		10
Azamethiphos	*Artemia salina*	Field study (dispersion from net pan enclosures)	24 h	mortality	---		10
Azamethiphos	*Gasterosteus aculeatus*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50}= 190 µg/L		10
Azamethiphos	*Lytechinus pictus*	Field study (dispersion from net pan enclosures)	20 min	fertilization	LC_{50} = 6840 g/L		10
Cypermethrin	*Vibrio fischeri*	Field study (dispersion from net pan enclosures)	15 min	luminescence	EC_{50}= >4950 µg/L		10
Cypermethrin	*Amphiporeia virginiana*	Field study (dispersion from net pan enclosures)	48 h	mortality	EC_{50} = 0.030 µg/L		10
Cypermethrin	*Gammarus spp*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50}= 0.036 µg/L		10
Cypermethrin	*Eohaustorius estuarius*	Field study (dispersion from net pan enclosures)	48 h	mortality	LC_{50}= >1µg/L		10
Cypermethrin	*Polydora cornuta*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50}= 27.8 µg/L		10
Cypermethrin	*Strongylocentrotus droebachiensis*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50} > 50 µg/L		10
Cypermethrin	*Brachionus plicatilis*	Field study (dispersion from	24 h	mortality	---		10

		net pan enclosures)					
Cypermethrin	*Artemia salina*	Field study (dispersion from net pan enclosures)	24 h	mortality	---		10
Cypermethrin	*Gasterosteus aculeatus*	Field study (dispersion from net pan enclosures)	96 h	mortality	LC_{50}= 8.1 µg/L		10
Cypermethrin	*Lytechinus pictus*	Field study (dispersion from net pan enclosures)	20 min	fertilization	LC_{50}= 2560 µg/L		10
Dichlorvos	*Artemia salina*	Laboratory settings	24 h	Mortality	LC_{50} (24 h)= 53700 µg/L LC_{50} (48 h) =32340 µg/L LC_{50} (72 h) =37990 µg/L	Sánchez-Fortún; Sanz-Barrera; Barahona-Gomariz, 1995 (doi: 10.1007/BF00196272)	11
Ivermectin	Ostracoda	Indoor microcosm	224 days	Abundance in meiobenthic community		Brinke et al. 2010 (doi:10.1016/j.aquatox.2010.04.000)	12
Ivermectin	Cladocera	Indoor microcosm	224 days	Abundance in meiobenthic community	NOEC (meiofauna,26 and 56 days) = 6.2 µg/L		12
Ivermectin	Nematoda	Indoor microcosm	224 days	Abundance in meiobenthic community	NOEC (community, 7-28 days) = 0.6 µg/L		12
Ivermectin	Tardigrada	Indoor microcosm	224 days	Abundance in meiobenthic community			12
Ivermectin	Oligochaeta	Indoor microcosm	224 days	Abundance in meiobenthic community			12

Table 2. Studies on the environmental fate of various veterinary antiparasitic investigating field and laboratory samples.

Compound	Location	Sample (Settings)	Quant. method	Key observations	Reference
Abamectin	São Paulo (Brazil)	Soil (laboratory settings)	HPLC-FLD	High sorption capacity, limited mobility. Dissipation mainly by microbial degradation (up to 4 days).	Dionisio, A. C. and Rath, S. 2016 (doi: 10.1016/j.chemosphere.2016.02.058)
Ivermectin	São Paulo (Brazil)	Soil (laboratory settings)	HPLC-DAD, HPLC-FLD	High sorption capacity to soils tested.	Rath et al. 2016 (doi: 10.1007/s11356-015-5787-6

				Difficult to desorb. Quickly degraded in both sandy and clay soil.	
Eprinomectin	Thessaloniki, Aridaia, Axios river (Greece)	Soil (laboratory settings)	HPLC-FLD	Eprinomectin is resistant to dissipation in the soils tested and in cattle manure.	Litskas et al. 2013 (doi: 10.1016/j.envint.2013.07.017)
Emamectin benzoate	Shetland Islands (Scotland)	Sediment near fish farms (field study)	LC-MS/MS	Found in 97% of samples. Above 0.763 µg/kg* in 7% of samples taken >100 m from the cages. *Environmental Quality Standard	Bloodworth et al. 2019 (doi: 10.1016/j.scitotenv.2019.02.430)
Teflubenzuron	Shetland Islands (Scotland)	Sediment near fish farms (field study)	LC-MS	Found at 3 out of 8 sights surveyed nearly 5 years after cessation of use. Above 0.763 µg/kg* in 36% of samples taken >100 m from the cages. *Environmental Quality Standard	Bloodworth et al. 2019 (doi: 10.1016/j.scitotenv.2019.02.430)
Fenbendazole	River Gos´cicina (Poland)	Water, sediment, and fish tissues (field study)	LC–MS/MS	Water: up to 85.7 ng/L (autumn). Residues in sediment and fish samples were mostly below quantification limits.	Wagil et al. 2015 (doi: 10.1016/j.chemosphere.2014.04.106)
Flubendazole	River Gos´cicina (Poland)	Water, sediment, and fish tissues (field study)	LC–MS/MS	Water: up to 39.2 ng/L (autumn). Sediment: up to 4.4 ng/g (autumn). Tissue samples: up to 38.5 ng/g (autumn). Residues in sediment and fish samples were mostly below quantification limits.	Wagil et al. 2015 (doi: 10.1016/j.chemosphere.2014.04.106)

Table 3. Other relevant studies: articles exploring aspects of pollution with veterinary antiparasitics outside the scope of toxicity testing or environmental fate.

Compound	Location	Stated objectives	Methodological information	Key findings	Reference
Ivermectin	European Union	1) To conduct an ERA for the parasiticide ivermectin 2) To show gaps and to propose improvements of the existing guidelines	Criteria: VICH 2000, 2004; EMEA 2008. Species and routes of administration. Integration of data from non-standardized studies.	Ivermectin is a substance of high concern. Guidance needed for higher-tier or tired studies. ERA for ivermectin needs reassessment.	Liebig et al. 2010 (doi: 10.1002/ieam.96)
Teflubenzuron	Oslo fjord (Norway)	To determine the uptake and depuration of drugs by *Mytilus edulis*	Measurement in collected field samples near fish farms. Laboratory exposure: 14 days of uptake (exposure)	Below limit of detection in field samples Kinetic bioconcentration factor (BCF): 1304.	Brooks et al. 2019 (doi: 10.1016/j.scitotenv.2018.11.212)

			+ up to 21 of depuration in clean seawater.	Calculated elimination half-life (t1/2): 4.7 days.	
Emamectin benzoate	Oslo fjord (Norway)	To determine the uptake and depuration of drugs by *Mytilus edulis*	Measurement in collected field samples near fish farms. Laboratory exposure: 14 days of uptake (exposure) + up to 21 of depuration in clean seawater.	Below limit of detection in field samples. Likely persistent in mussel tissue. Kinetic bioconcentration factor (BCF): 49. Calculated elimination half-life (t1/2): 14 days.	Brooks et al. 2019 (doi: 10.1016/j.scitotenv.2018.11.212)
Deltamethrin	Oslo fjord (Norway)	To determine the uptake and depuration of drugs by *Mytilus edulis*	Measurement in collected field samples near fish farms. Laboratory exposure: 14 days of uptake (exposure) + up to 21 of depuration in clean seawater.	Below limit of detection in field samples. Kinetic bioconcentration factor (BCF): 2516. Calculated elimination half-life (t1/2): 0.87 days.	Brooks et al. 2019 (doi: 10.1016/j.scitotenv.2018.11.212)

Table 4. Most and least sensitive organisms (as defined by EC_{50} values) for compounds tested in more than one test organism. Ref: reference number.

Compound	No. test organism entries	Lowest EC_{50}	Most sensitive organism	Highest EC_{50}	Last sensitive organism	Ref.
Ivermectin	27	0.59 µg/L	*Daphnia magna*	1961 µg/L	*Brachionus calyciflorus*	3
Fenbendazole	14	16.7 µg/L	*Daphnia magna*	>8000 µg/L	*Brachionus calyciflorus*	3
Flubendazole	13	21.9 µg/L	*Dugesia gonocephala*	>8000 µg/L	*Brachionus calyciflorus*	3
Cypermethrin	10	0.030 µg/L	*Amphiporeia virginiana*	>4950 µg/L	*Vibrio fischeri*	10
Azamethiphos	10	>20 µg/L(LC_{50})	*Eohaustorius estuarius*	11000 µg/L(LC_{50})	*Vibrio fischeri*	10
Trichlorfon	4	0.29 µg/L	*Daphnia magna*	274.5 mg/L	*Pseudokirchneriella subcapitata*	6
Abamectin	4	0,25 µg/L	*Daphnia magna*	690 µg/L	*Vibrio fischeri*	9
Doramectin	4	0.0637 µg/L	*Daphnia magna*	Not reached	*Vibrio fischeri,*	5

			Scenedesmus vacuolatus, Lemna minor

CONCLUSÕES

As informações obtidas por meio de estudos experimentais e de revisão sistemática demonstram a importância tanto da moxidectina quanto dos antiparasitários de uso veterinário em geral enquanto potenciais poluentes ambientais. Foi estabelecido que os efeitos de antiparasitários de uso veterinário sobre ambientes aquáticos são predominantemente baseados em ensaios de toxicidade com organismos invertebrados, mas que ainda existem lacunas com relação a seus mecanismos de toxicidade e condições de exposição no meio ambiente.

Ademais, as exposições de embriões de zebrafish à moxidectina resultaram em um padrão de neurotoxicidade e estresse oxidativo. Através de análises biológicas e químicas, foi possível observar que o atraso na eclosão causado pela moxidectina ocorre sob a influência de efeitos neurotóxicos, particularmente ligados ao aumento da atividade da AChE e à acumulação nas regiões da cabeça e olhos. No entanto, não pode ser descartada a influência de mecanismos diversos associados ao estresse oxidativo, bem como a ação de efeitos metabólicos sem relação específica com os efeitos observados, mas que contribuem para aumentar a carga alostática. Além disso, as análises químicas revelaram que a moxidectina concentra-se forte e rapidamente no embrião, demonstrando que mesmo os compostos com degradação rápida podem ser capazes de alcançar organismos não-alvo de forma eficaz.

Portanto, o presente trabalho é uma clara demonstração de que as questões sobre a poluição ambiental e suas ramificações podem ser respondidas por meio de abordagens diversas. Os resultados também fornecem subsídios para a avaliação dos riscos impostos a ambientes aquáticos pelo uso da moxidectina, uma vez que foram caracterizados os seus efeitos neurotóxicos e seu potencial de acumulação em embriões de zebrafish.

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ANEXO I – Documento de autorização da Comissão de Ética

Proposta: Avaliação dos impactos à vida aquática e à saúde humana de poluentes emergentes no Brasil: uso do peixe-zebra (*Danio rerio*) como sistema modelo.

Protocolo No.: 8881290419.

Órgão emissor: Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba (CEUA/UFPB).

Vigência da proposta: 08/2019 a 07/2021.

Universidade Federal da Paraíba

Comissão de Ética no Uso de Animais
Reitoria

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação dos impactos à vida aquática e à saúde humana de poluentes emergentes no Brasil: uso do peixe-zebra (Danio rerio) como sistema modelo", protocolada sob o CEUA nº 8881290419 (ID 000729), sob a responsabilidade de **Davi Felipe Farias** *e equipe; Igor Cauê Alves Araruna; Marília da Guia Flor da Silva; Marta Silva Muniz; Rafael Xavier Martins; Juliana Alves da Costa Ribeiro* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba (CEUA/UFPB) na reunião de 05/07/2019.

We certify that the proposal "Evaluation of the impacts to aquatic life and human health of emerging pollutants in Brazil: use of zebrafish (Danio rerio) as a model system", utilizing 444 Fishes (males and females), protocol number CEUA 8881290419 (ID 000729), under the responsibility of **Davi Felipe Farias** *and team; Igor Cauê Alves Araruna; Marília da Guia Flor da Silva; Marta Silva Muniz; Rafael Xavier Martins; Juliana Alves da Costa Ribeiro* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Paraíba (CEUA/UFPB) in the meeting of 07/05/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 08/2019 a 07/2021 Área: Ciências Biológicas

Origem:	Unidade de Produção de Organismos Modelo Não Convencionais (UniPOM)						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	1 a 3 horas	N:	444
Linhagem:	Danio rerio			Peso:	0 a 0 g		

Local do experimento: Todos os experimentos serão realizados no Laboratório de Avaliação de Risco de Novas Tecnologias que divide infraestrutura com a Unidade de Produção de Organismos Modelo Não Convencionais, Depto. de Biologia Molecular, Campus I, UFPB.

João Pessoa, 05 de novembro de 2020

Profa. Dra. Jailane de Souza Aquino
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal da Paraíba

Prof. Dr. Carlos Augusto Alanis Clemente
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal da Paraíba