INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA GOIANO – CAMPUS RIO VERDE
DIRETORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM AGROQUÍMICA

ENSAIOS ECOTOXICOLÓGICOS UTILIZANDO PLANÁRIAS *(Girardia tigrina)* COMO BIOINDICADORES DO EFEITO LETAL E SUBLETAL DE FIPRONIL E CLORPIRIFÓS

Autora: Eloisa Borges dos Reis
Orientador: Dr. Althiéris de Souza Saraiva
Coorientadora: Dra. Fernanda dos Santos Farnese
Coorientadora: Dra. Marilene Silva Oliveira

RIO VERDE – GO
FEVEREIRO – 2021

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Autora: Eloisa Borges dos Reis
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TITULAÇÃO: Mestre em Agroquímica - Área de Concentração Agroquímica

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Dr.ª Aline Silvestre Pereira Dornelas
Avaliadora externa - Foco Ambiental Consultoria

Prof. Dr. Dener Márcio da Silva Oliveira
Avaliador interno - IF Goiano / Campus Posse

Prof. Dr. Althiéris de Souza Saraiva
Presidente da Banca - IF Goiano / Campus Campos Belos

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"a quem seja a glória pelos séculos dos séculos. Amém!" Gálatas 1:5

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BIOGRAFIA DA AUTORA

Eloisa Borges dos Reis, filha de Eloir Gonçalves dos Reis e Ivana Borges Pereira Reis, natural de Piranhas-GO, nasceu em 21 de junho de 1996.

Em 2014 ingressou no curso de bacharel em Engenharia Ambiental, pela Universidade de Rio Verde – UniRV, Campus Caiapônia. Graduou-se em janeiro de 2019.

Neste mesmo ano (2019), iniciou o mestrado pelo Programa de Pós-Graduação em Agroquímica – PPGAq, no Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Rio Verde, sob orientação do prof. Dr. Althiéris de Souza Saraiva e coorientação das professoras Dra. Fernanda dos Santos Farnese e Dra. Marilene Silva Oliveira, concluindo –o em fevereiro de 2021.

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LISTA DE SIGLAS E ABREVIATURAS

ASTM - American Standard Test and Materials

CL_{50} – Concentração Letal

K_{ow} - Coeficiente de partição octanol/água

LOEC - Lowest Observed Effect Concentration

NOEC - No Observed Effect Concentration

PET - Poli Tereftalato de Etila

$T^{1/2}$ - tempo de meia-vida

RESUMO

REIS, ELOISA BORGES. Dissertação apresentada ao Instituto Federal Goiano – Câmpus Rio Verde – GO, como parte das exigências da Pós-Graduação – Mestrado em Agroquímica. Fevereiro 2021. **Ensaios ecotoxicológicos utilizando planárias (*Girardia tigrina*) como bioindicadores do efeito letal e subletal de Fipronil e Clorpirifós.** Orientador: Dr. Althiéris de Souza Saraiva, Coorientadoras: Dra. Fernanda dos Santos Farnese. Dra. Marilene Silva Oliveira.

O aumento significativo da população mundial ocasiona, consequentemente, maior demanda por alimentos. O avanço nas atividades agropecuárias brasileiras promove aumento de novas técnicas que proporcionam maior produtividade e menor perda no processo produtivo, ao passo que pode resultar em um sistema totalmente dependente da utilização de agrotóxicos. Inúmeros ingredientes ativos de agrotóxicos têm sido reportados para atingir, ou têm sido encontrados nos ecossistemas de água doce sendo que a toxicidade destes pesticidas podem, potencialmente, afetar o ecossistema aquático. Contudo, o potencial de um agrotóxico para atingir águas superficiais depende, entre outros fatores, das características físico-químicas do composto assim como também da intensidade e periodicidade em que é aplicado. Dentre os inseticidas amplamente utilizados na produção agropecuária, tem-se o Fipronil e o Clorpirifós. Neste contexto, as planárias se apresentam como boas candidatas a organismos bioindicadores de contaminação ambiental. Diante disso, o estudo objetiva abordar a toxicidade aguda e crônica de inseticidas a base de Fipronil e Clorpirifós, por meio de ensaios ecotoxicológicos para avaliação de locomoção, regeneração e reprodução da planária de água doce tropical *Girardia tigrina*. Para os testes realizados com Fipronil não houve concentração que causasse efeito agudo, portanto, o composto ocasionou efeitos crônicos no que diz respeito ao atraso da locomoção, regeneração e reprodução das planárias. Não obstante, o Clorpirifós ocasionou efeito letal e subletal (locomoção, regeneração e reprodução) das planárias em concentrações ambientalmente relevantes muito mais inferiores ao que reportado na literatura científica.

Palavras-chaves: Bioindicadores, Inseticidas, Efeito agudo, Efeito crônico

ABSTRACT

REIS, ELOISA BORGES. Dissertation presented to the Federal Institute of Goiás - Campus Rio Verde - GO, as part of the requirements of the Post-Graduation - Master in Agrochemistry. February 2021. **Ecotoxicological tests using planarians (*Girardia tigrina*) as bioindicators of the lethal and sublethal effect of Fipronil and Chlorpyrifos.** Advisor: Dr. Althiéris de Souza Saraiva, Co-supervisors: Dra. Fernanda dos Santos Farnese. Dra. Marilene Silva Oliveira.

The significant increase in the world population causes a greater demand for food, consequently. The advance in the Brazilian agricultural activities promotes an increase in new techniques that provide greater productivity and less loss in the production process, meanwhile it may result in a system which is totally dependent on the use of pesticides. Numerous pesticides active ingredients have been reported to target, or have been found in freshwater ecosystems, also meaning that the toxicity of these pesticides may potentially affect the aquatic ecosystem. However, the potential of a pesticide in order to reach water surface, depends, among other factors, on the physical-chemical characteristics of the compound and the intensity and periodicity in which it is applied. Among the insecticides widely used in agricultural production, there are Fipronil and Chlorpyrifos. Relaying this context, planarians present themselves as good candidates for bioindicators of environmental contamination. Therefore, the study aims to address the acute and chronic toxicity of insecticides based on Fipronil and Chlorpyrifos, through ecotoxicological tests to assess locomotion, regeneration and reproduction of the tropical freshwater planaria *Girardia tigrina*. For the tests carried out with Fipronil, there was no concentration that would cause an acute effect, therefore, the compound caused chronic effects regarding to the delay of locomotion, regeneration and reproduction of planarians.However, it was noticed that Chlorpirifós caused a lethal and sublethal effect (locomotion, regeneration and reproduction) of planarians in environmentally relevant concentrations considered to be much lower than reported in the scientific literature.

Keywords: Bioindicators, Insecticides, Bioindicators, Acute effect, Chronic effect

1. INTRODUÇÃO GERAL

1.1 Produção Agropecuária e o Uso de Agrotóxicos

O Brasil possui diversidade na produção agrícola e pecuária, dado ser de clima tropical e temperado, ao passo que as regiões Sul e Centro-Oeste destacam-se no cenário da produção agropecuária nacional (IBGE, 2017). Adicionalmente, o rápido crescimento do setor agropecuário brasileiro, principalmente nas duas últimas décadas, está relacionado, entre outros fatores, à reformas na economia, modificações e liberalização de práticas agriculturáveis (OECD-FAO, 2015).

A produção de grãos no último ano no Brasil, fechou o ciclo com 253,7 milhões de toneladas, apresentando um aumento de 4,8% em comparação com a safra retroativa (CONAB, 2020). As principais culturas produzidas no país são: soja, milho, arroz e algodão. Menciona-se ainda que a produção pecuária nacional merece destaque, uma vez que para o ano de 2019 a destinação de áreas para pastagens foi de 158,6 milhões de hectares, correspondente a 18,6% de todo o território nacional (MAPA, 2019).

Por outro lado, ao mesmo tempo em que a produção agropecuária brasileira cresce, a utilização de produtos químicos é intensificada na agricultura e culturas de interesse zootécnico, fato este que tem levado o Brasil a liderar o ranking mundial dos países que mais utilizam agrotóxicos (VASCONCELOS, 2018). A Lei 7.802/1989 define agrotóxicos como insumos de origem química, física ou biológica, utilizados nos setores agropecuário e florestal, com o objetivo de modificar componentes da fauna ou flora, cuja finalidade é conservar os organismos do efeito nocivo de seres vivos (BRASIL, 1989).

Adicionalmente, o elevado crescimento populacional demanda maior produção de alimentos e, por sua vez, o aumento do uso de agrotóxicos na produção agropecuária tem sido considerado essencial para que haja produtividade satisfatória. Neste contexto, o setor agropecuário, ao longo das últimas décadas, tornou-se dependente do uso de agrotóxicos, ao passo que os princípios da sustentabilidade não têm acompanhado este rápido avanço na agricultura (PAPADAKIS et al., 2018).

Mesmo com o incentivo, as práticas de manejo sustentável, a biodiversidade têm sido afetadas pelas práticas não conservacionistas no setor agropecuário, como é o caso do uso intensivo de agrotóxicos que podem ocasionar contaminação da biodiversidade (OECD, 2008). De fato, na contramão do acelerado crescimento do setor agropecuário, a biodiversidade tem sofrido impactos, pelo uso intensivo de princípios ativos de agrotóxicos que, por vezes, são proibidos em países desenvolvidos devido aos potenciais de contaminação do solo, do ar, da água e da saúde humana (CRUZEIRO et al., 2017; FIORESSI et al., 2019).

Neste cenário, a produção agropecuária brasileira tornou-se dependente dos inúmeros ingredientes ativos de agrotóxicos que são utilizados com vistas ao aumento da produção e redução de perdas envolvidas no processo produtivo. Adicionalmente, a superdosagem de agrotóxicos pode provocar diversos danos ao agroecosssitema como: resistência das pragas aos produtos químicos, surgimento de pragas secundárias e ressurgimento de pragas (SMITH, 1970). Dessa forma, os impactos ocasionados por defensivos agrícolas podem atingir de forma direta os organismos não-alvo (aquático e terrestre), e de modo indireto afeta os insetos benéficos, os seres humanos, através da contaminação da água, solo, ar e alimentos, ocasionando impactos a toda a biodiversidade (AKTAR; SENGUPTA; CHOWDHURY, 2009).

De fato, o uso intensivo e periódico de agrotóxicos, nas diversas fases de produção agropecuária tem elevado potencial para atingir ecossistemas adjacentes às áreas de produção, a exemplo da contaminação de ecossistemas de água doce (BALDANTONI et al., 2018; CHEN et al., 2018; HOSSAIN; ROY, 2018; RIBEIRO et al., 2019; BARTLETT et al., 2019).

1.2 Efeitos de Agrotóxicos em Ecossistemas de Água Doce

O uso insustentável da terra associado ao manejo inadequado das bacias hidrográficas pode interferir nos parâmetros quali-quantitativos da água (APARECIDO et al., 2016). De acordo com aproximadamente 90% dos Planos de Gestão de Bacias Hidrográficas, a agricultura é um dos principais setores que ocasiona impactos potenciais na qualidade e disponibilidade de água (EC, 2012). Ademais, as bacias rurais são caracterizadas pelo uso do solo, como áreas de agricultura e pastagem, sendo que o manejo inadequado dessas áreas resulta em impactos na qualidade e disponibilidade da água.

O Brasil é um país que possui abundância em água, porém, o desenvolvimento econômico aliado ao aumento da densidade populacional, proporcionaram impactos nos recursos hídricos, ocasionados, principalmente devido à intensificação das atividades agropecuárias e industriais (SARAN et al., 2018; NASCIMENTO et al., 2018; SILVA; GONÇALVES; MORALES, 2018). Neste sentido, as águas superficiais podem sofrer alterações pela contaminação de agrotóxicos em decorrência da lixiviação e escoamento superficial, devido à superdosagem e utilização indevida destes produtos; fatos estes que podem ocasionar impactos em ecossistemas aquáticos (CRUZEIRO et al., 2017; SANTANA et al., 2018; SOLIS et al., 2018).

A Resolução Conama 357/2005 classifica os corpos d'água em Águas Doces, Salinas e Salobras, e subdivide-os em classes especiais, 1, 2, 3 e 4, delimitando os seus respectivos usos, como abastecimento humano (após desinfecção; tratamentos simplificado, convencional, ou avançado), irrigação, harmonia paisagística, pesca, dessedentação de animais, bem como, estabelece limites para elementos e substâncias que estão inseridos na água, tanto para consumo humano, quanto para irrigação, pesca e equilíbrio do ecossistema aquático (BRASIL, 2005). Contudo, a legislação que regula o uso de agrotóxicos no Brasil é diligentemente e diversa das políticas públicas seguidas nos países desenvolvidos, como por exemplo a União Europeia. Gonçalves (2016), que reporta que valores de resíduos de pesticidas autorizados no Brasil são muito superiores aos limites estabelecidos pela União Europeia. Sabe-se também que, compostos químicos permitidos para uso em território brasileiro são terminantemente proibidos na União Europeia.

Os ecossistemas aquáticos de água doce têm sido consideravelmente afetados, pela pressão do uso intensivo de agrotóxicos em áreas de produção agropecuária, principalmente, em áreas de produção próximas aos recursos hídricos (BARBOSA; SOLANO; UMBUZEIRO, 2015). Neste contexto, diversos estudos de ocorrência e quantificação de ingredientes ativos de agrotóxicos têm sido reportados na literatura científica internacional.

De modo geral, os estudos mostram que o uso intensivo de agrotóxicos pode ocasionar danos aos ecossistemas e, consequentemente, afeta a biodiversidade (PAPADAKIS et al., 2018; KNILLMANN et al., 2018; MAMUN et al., 2019; ROBINSON et al., 2019; TSABOULA et al., 2019).

Menciona-se ainda que os estudos ecotoxicológicos têm demonstrado a toxicidade de agrotóxicos sobre organismos de água doce não-alvo, tanto vertebrados (GOODIER; PROPPER, 2016; METER et al., 2018; CASADO et al., 2019; RESENDIZ et al., 2019; ROSALES et al., 2019), quanto invertebrados (BARMENTLO et al., 2018; MONTEIRO et al., 2018; RABY et al., 2018; RICO et al., 2018; AMORIM et al., 2019).

Estes estudos ecotoxicológicos com organismos modelo e/ou bioindicadores de contaminação ambiental tornam-se dados científicos relevantes, uma vez que contribuem com a análise de risco ecológico de determinado ingrediente ativo, através de ensaios de toxicidade aguda e crônica a nível subindividual, nível individual e outros níveis de organização biológica (RABY et al., 2018; CHARRY et al., 2019; LI et al., 2019). Cabe ressaltar ainda, que podem ser encontradas maiores concentrações de agrotóxicos nos seres aquáticos do que na água, pois, estes contaminantes podem se acumular nos órgãos e tecidos destes organismos (BORRELL et al., 2016).

1.3 Impactos do Inseticida Fipronil no Ambiente

O Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis (IBAMA) proibiu a aplicação aérea de compostos químicos à base de Fipronil, além de Imidacloprido, Tiametoxam e Clotianidina em qualquer tipo de plantação haja visto que estes compostos estão em processo de reavaliação toxicológica, devido ao seu elevado efeito tóxico para organismos não-alvo, mais especificamente as abelhas. Continuamente, tem se associado a morte destes insetos com a aplicação aérea dos referidos inseticidas em diversas regiões do Brasil, fato este que culminou a proibição deste ato. (IBAMA, 2018).

Ademais, a legislação brasileira não estabelece limites permitidos para o Fipronil em ecossistemas naturais. O potencial de Fipronil atingir ecossistemas aquáticos pode estar relacionado às suas características físico-químicas, a exemplo de solubilidade em água, coeficiente de partição octanol/água, coeficiente de partição solo/água e, potencial de lixiviação (Tabela 1).

Tabela 1 – Propriedades físico-químicas de Fipronil.

Pesticida	Solubilidade em água mg/L	Log K_{ow}	$T^{1/2}_{Campo}$ (dias)	$T^{1/2}_{Água\ e\ Sedimento}$ (dias)	Potencial de Lixiviação	Concentração ambientalmente relevante µg/L
Fipronil	3,78[a]	3,75 [a]	54 [a]	68 [a]	2,45 [a]	26,2[b]

K_{ow} = Coeficiente de partição octanol/água; $T^{1/2}$ = tempo de meia-vida; [a] Valores obtidos a partir da base de dados Pesticide Properties DataBase (PPDB - University of Hertfordshire); [b] Albuquerque et al. (2016) *Índice GUS (Groundwater Ubiquity Score) ≥ 2,8 indica alta capacidade de lixiviação, ≤ 1,8 = Baixo potencial de lixiviação, e < 0 = Potencial de lixiviação muito baixo (GOSS, 1992).

1.4 Impactos do Inseticida Clorpirifós no Ambiente

O Clorpirifós é um inseticida amplamente utilizado a nível mundial (JOHN; SHAIKE, 2015). Em 2013, países da União Europeia proibiram a utilização doméstica do Clorpirifós, pois foi verificado que este provoca danos prejudiciais a organismos não alvo (EFSA, 2013). No Brasil, o Clorpirifós está em reavaliação toxicológica, desde 2019, pela Agência Nacional de Vigilância Sanitária (ANVISA), devido à evidência de causar risco à saúde humana (ANVISA, 2019).

As características físico-químicas do Clorpirifós (Tabela 2), a saber, o potencial de lixiviação, a solubilidade em água e o tempo de meia vida em solo e água, também podem estar relacionados ao potencial do Clorpirifós atingir corpos hídricos.

Tabela 2 – Propriedades físico-químicas do Clorpirifós.

Pesticida	Solubilidade em água mg/L	Log K_{ow}	$T^{1/2}_{Campo}$ (dias)	$T^{1/2}_{Água\ e\ Sedimento}$ (dias)	Potencial de Lixiviação	Concentração ambientalmente relevante µg/L
Clorpirifós	1,05[a]	4,70 [a]	386 [a]	36,5 [a]	0,58 [a]	37,3[b]

K_{ow} = Coeficiente de partição octanol/água; $T^{1/2}$ = tempo de meia-vida; [a] Valores obtidos a partir da base de dados Pesticide Properties DataBase (PPDB - University of Hertfordshire); [b] Hasanuzzaman et al. (2018) *Índice GUS (Groundwater Ubiquity Score) ≥ 2,8 indica alta capacidade de lixiviação, ≤ 1,8 = Baixo potencial de lixiviação, e < 0 = Potencial de lixiviação muito baixo (GOSS, 1992).

1.5 Planárias como Organismo Bioindicador de Contaminação Ambiental

Dentre os organismos bioindicadores de contaminação aquática pelo uso de pesticidas têm-se as planárias, as quais são difundidas em águas doces tropicais e são bons candidatos para avaliação dos efeitos tóxicos de inseticidas a base de Fipronil e Clorpirifós. Estes organismos são considerados bons organismos bioindicadores por viverem em ambientes de água doce não poluída (NOREÑA et al., 2015; SCHOCKAERT et al., 2008; VILA-FARRÉ; RINK, 2018) e por apresentarem características biológicas interessantes para estudos ecotoxicológicos, já que o sistema nervoso destes organismos compartilha muitas características com os vertebrados em termos de morfologia e fisiologia celular (REDDIEN. ALVARADO, 2004; BUTTARELLI et al., 2008; WU; LI, 2018).

Estudos que utilizam organismos aquáticos em testes, como por exemplo invertebrados, são úteis para pesquisas em diversas áreas, pois por intermédio dos mesmos, é possível analisar efeitos a nível de população e comunidade.

Nos últimos anos houve um significativo aumento de pesquisas que utilizam organismos aquáticos, em decorrência da rapidez e eficácia dos seus testes. Ademais, apresentam características biológicas e toxicológicas mais simples de serem estudadas. do que por exemplo, estudos que envolvam mamíferos pois tais estudos demandam mais tempo e são mais difíceis de se analisar. (WU; LI, 2018).

Cabe ressaltar ainda que planárias ocupam diferentes níveis tróficos nos ecossistemas aquáticos; são predadores de invertebrados bentônicos encontrados em ecossistemas de água doce (e. g. larvas de insetos), e ao mesmo tempo servem de alimento para outros invertebrados e vertebrados predadores (OVIEDO et al., 2008; HAGSTROM et al., 2015; RODRIGUES et al., 2016).

Menciona-se ainda que planárias são hermafroditas, com reprodução sexuada ou assexuada (por fissão binária) (NOREÑA et al., 2015), de fácil obtenção e manutenção em laboratório, principalmente, devido ao baixo custo e possibilidade de avaliação de diferentes parâmetros fisiológicos (regeneração e reprodução) e comportamentais (locomoção e alimentação) (DORNELAS et al., 2020; LI, 2014; SIMÃO et al., 2021; SHEIMAN; KRESHCHENKO, 2015; OFOEGBU et al., 2016; OFOEGBU et al., 2019; OFOEGBU et al., 2019; SARAIVA et al., 2018; SARAIVA et al., 2020; LÓPEZ et al., 2019).

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CHAPTER I

Toxicity of Fipronil on Tropical Freshwater Planarian *Girardia tigrina*

ABSTRACT

Fipronil is an insecticide of the pyrazole group which is widely used to insect control. In places where the - Fipronil is used in a large-scale application, there is a potential effect on water surface contamination, damage to non-target organisms, and potential impacts on aquatic ecosystems. Among the bioindicators organisms of aquatic contamination, there are planarians, which are spread in tropical freshwaters and are good candidates to assess the toxic effects of insecticides based on Fipronil. Studies addressing the lethal and sublethal effects of Fipronil in aquatic invertebrates are still scarce. Thus, the study aimed to analyze the lethal and sublethal concentrations of Fipronil in planaria of the species *Girardia tigrina*. Regarding the lethal effect, no concentration caused the death of the organisms. However, in the case of chronic effects, there was a significant delay in locomotion (LOEC - 6.25 mg a.i/L), head regeneration (LOEC - 1.56 mg a.i.L^{-1}), regeneration of the auricles and photoreceptors (LOEC – 3.13 mg a.i.L^{-1}), and reproduction (Fecundity - LOEC 12.5 mg a.i.L^{-1}). The results of our study demonstrate the importance of using planarians as potential bioindicators in relation to environmental contamination by Fipronil, with behavioral and physiological changes in similiar procedures considering what has been reported by other freshwater organisms model in ecotoxicology.

Keywords: Lethal effect, Pesticides, Phenylpyrazole, Planarians, Sublethal effect,

1. INTRODUCTION

The significant increase of the global population leads to a greater demand for food, consequently. Then it´s noticed that the Brazilian agricultural activity has expanded considerably, providing improvements in income and, thus, it has contributed to the base of the economy, in the scenario of agribusiness (OECD-FAO, 2015). Moreover, the tropical climate and the abundance of water resources are factors that favor agri-food growth in the country (IBGE, 2017). Currently, Brazil has been recognized worldwide for the high potential of agricultural and livestock production (OCDE, 2018).

The advance in agricultural activities supplies and the ncrease of the new techniques which leads to more productivity and the reduction of losses in the production process may result in a system which is dependent on the use of pesticides (PAPADAKIS et al., 2018). The use of these compounds, especially, when they are used incorrectly and without proper technical support, may impact natural ecosystems, and their biodiversity (water, soil, air, and human health as well) (HE et al., 2019; QU et al., 2019; ANH et al., 2019; SABARWAL; KUMAR; SINGH, 2018). The toxicity of pesticides depends on the active ingredient component in each product, however, the effects caused by its use, can occur in short (acute effects), medium (subchronic effects), and long (chronic effects) times, interfering in the life cycle of organisms (IBAMA, 2010; KORKMAZ; GÜNGÖRDÜ; OSMEN, 2018; ZHU et al., 2018).

Numerous active ingredients of pesticides have been reported to reach or have been found in freshwater ecosystems, and the toxicity of these pesticides can affect the aquatic ecosystem performance, potentially. In places where the insecticide Fipronil is used on a large scale, there is a potential effect on surface water contamination, damage to non-target organisms, and potential impacts on aquatic ecosystems (BEDIENT, et al., 2005). The insecticides based on Fipronil used in agriculture and livestock can reach freshwater ecosystems and the environmentally relevant concentration in surface water is 26.2 $\mu g\ L^{-1}$ (ALBUQUERQUE et al., 2016). Fipronil is an insecticide of the phenylpyrazole group that is used in the agricultural sector. According to studies, this insecticide causes the death of insects due to the effect of paralysis and neural excitation (REGAN et al., 2017).

In the ecotoxicological field, freshwater planarians of the species *Girardia tigrina* (Girard, 1850) (Paludícola: Dugesiidae), are reported as bioindicator organisms of environmental contamination, therefore, their behavior (locomotion and feeding) and capacity for regeneration and reproduction have been studied to assess the toxicity of pesticides (DORNELAS et al., 2020; OFOEGBU et al., 2019; OFOEGBU et al., 2019; SARAIVA et al., 2020; SIMÃO et al., 2021). Planarians are organisms which are easy to monitor at the laboratory; they are predatory invertebrates, as well as food for other organisms (prey); factors that turn them candidates for test organisms in ecotoxicology (LÓPEZ et al., 2019; SARAIVA et al., 2018; VILA-FARRÉ; RINK, 2018). In recent years, international scientific research has used planarians in laboratory tests in several areas such as ecotoxicology, pharmacy, and health, due to the practicality and ease of the tests, when compared to tests using mammals (WU; LI, 2018). Tests with planarians are faster due to their biological characteristics, such as high regeneration capacity.

Thus, the aim of this study was to evaluate the lethal and sublethal toxicity of Regent 800 WG (Fipronil insecticide) on the freshwater planarian *G. tigrina*.

2. MATERIALS AND METHODS

2.1 *Girardia tigrina*

Planarians *(G. tigrina)* were obtained from the selected culture at the Ecotoxicology Laboratory at the Federal University of Tocantins – UFT (Research Group in Applied and Functional Ecology, Gurupi Campus). At Plant Metabolism and Ecotoxicology Laboratory of IF Goiano – Campus Rio Verde, planarians were grown in American Standard Test and Materials aqueous media (ASTM) (ASTM, 1980), under a controlled temperature at 22 ± 1 °C, in the dark.

Once a week, the planarians were fed, *ad libitum*, with bovine liver, and the medium was renewed one or two hours after fed. The organisms were deprived of food once a week before experiments, to avoid contamination by digesting food and to certify homogeneity in response to toxicity (OVIEDO et al., 2015).

2.2 Preparation of Insecticide Based on Fipronil

A stock solution of insecticide based on Fipronil was prepared using 5000 mg of the Regent 800 WG® granulated of equivalent compound of fipronil/L, in distilled water.

The stock solution was kept in absence of light at 4 °C to avoid the degradation of the active ingredient. The experimental solution was prepared by the dilution of stock solution using ASTM medium.

2.3 Lethal Toxicity Assay

The determination of acute concentration that is lethal to 50% of organisms population (LC_{50}) was carried out by using Fipronil insecticide at seven different concentrations (100, 150, 225, 338, 507, 760 e 1140 mg L^{-1}), and the ASTM medium was used as control. Assays were performed in polyethylene terephthalate (PET) flasks containing 20 mL of experimental solution at 22 ± 1 °C. For each concentration, five replicates were used containing four planarians (1.0 ± 0.2 cm full-length). The organisms were kept for 48 hours in a static system, in the absence of light. All test dilutions were prepared using ASTM medium (adapted from SARAIVA et al., 2018; LÓPEZ et al., 2019).

2.4 Sublethal Toxicity Assay

Assessment of locomotion and regeneration was performed by using planarians of 1.0 ± 0.2 cm full-length, which were exposed for eight days at different; concentrations of Fipronil insecticide with the following nominal concentrations: 1.56, 3.13, 6.25, 12.5, 25 mg L^{-1}, and the control experiment (ASTM). The tests were carried out at a temperature of 22 ± 1 ° C, in a static system and in absence of light. Planarians were not fed for one week before ecotoxicological assays. The exposure was carried out with a group of 30 organisms, divided into three replicates (with 10 planarians per replicate) per treatment, in glass beakers containing 100 mL of experimental solution. After the fourth and eighth day of exposure, the experimental solutions were renewed with the respective test concentrations, where the planarians were allocated, for the evaluation of the effects on locomotion and regeneration (adapted from SARAIVA et al., 2018; LÓPEZ et al., 2019).

2.4.1 Planarian Locomotor Velocity - *p*LMV

The planarian locomotor velocity (*p*LMV) was evaluated by using a recipient covered with a lined sheet of paper (lines spaced at 0.5 cm) adding up the volume of ASTM medium in the amount that favored the locomotion of organisms. In order to allow their free movement, the planarians were placed in the center of the lined sheet of paper. After thirty seconds of adaptation, the planarians were monitored by the centimeters traveled for two minutes period (adapted from LÓPEZ et al., 2019; PESTANA; OFOEGBU, 2021; SARAIVA et al., 2018).

2.4.2 Regeneration

In the regeneration step, fifteen planarians were decapitated by a single precise cut behind the auricle, using a previously sterilized scalpel blade, for each concentration. After decapitation, the planarians were individually transferred to a polyethylene terephthalate (PET) flask containing 20 mL of ASTM medium. Then, the regeneration was analyzed by monitoring the number of hours (at 24 hours each individual was checked separately) until the formation of new photoreceptors and auricles, as well as the complete regeneration of the head, by using a magnifying glass Magnifier Lamp (adapted from LÓPEZ et al., 2019; PESTANA; OFOEGBU, 2021; SARAIVA et al., 2018).

2.4.3 Reproduction

Adult planarians at the beginning of the reproductive age (1.5 ± 0.1 cm full-length) were exposed for 4 weeks to assess fertility and for 3 weeks to assess fecundity (KNAKIEVICZ et al., 2006). They were exposed at 5 different concentrations of Fipronil (1.56, 3.13, 6.25, 12.5, 25 mg L^{-1}), and control treatment (only ASTM medium), in triplicate, each replicate containing 10 organisms. These organisms were exposed to 100 mL of experimental solution in PET flasks. Weekly, after feeding the organisms with bovine liver, the solutions of each concentration were replaced by new solutions, (*ad libitum*). The experiment was carried out at a temperature of 22 ± 1 ° C, in absence of light, and observed daily. Each deposited cocoon was placed in a PET container with 20 mL of experimental solution. Fertility was assessed by the number of cocoons produced per day, divided by the number of exposed planarians. The fecundity rate was determined by the number of offspring planarias (planaria that was born from the cocoons), divided by the number of deposited cocoons (adapted from DORNELAS et al., 2020; KNAKIEVICZ et al., 2006).

2.5 Statistical analysis

The sublethal toxicity parameters were evaluated by analysis of variance (ANOVA) and successively Dunnett's post-hoc test was applied to assess whether there are significant differences between treatments. In order to verify whether the data were in accordance with ANOVA's assumptions, locomotion and reproduction; tests were analyzed for homogeneity of variances and normality, using the Bartlett and Kolmogorov-Smirnov tests, respectively. The regeneration tests were not in accordance with the assumptions of ANOVA analysis; therefore, it was necessary to use nonparametric statistics using the Kruskal Wallis test (Dunns post-hoc test). Statistical analysis were performed by using the GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, California. EUA).

3. RESULTS

3.1 Lethal effect of Fipronil on planarians

To evaluate the lethal effect of Fipronil in the organisms, several acute toxicity tests were carried out, however, there was no mortality of planarians exposed to the analyzed concentrations, as well, there was no mortality of organisms in the control treatments. Thus, it is considered that the lethal effect (CL_{50}) of Fipronil (Regent 800 WG®) for *G. tigrina* is above 1000 mg L^{-1}.

3.2 Sublethal effects of Fipronil on planarians

The planarians locomotor velocity of the (*p*LMV) (*G. tigrina*) decreased significantly after exposure to Fipronil (Regent 800 WG®), when compared to the control treatment ($F_{(5, 84)} = 11.1$; $p < 0.0001$), presenting a NOEC (No Observed Effect Concentration) of 3.13 mg i.a. L^{-1}, and a LOEC (Lowest Observed Effect Concentration) of 6.25 mg i.a. L^{-1} (Fig. 1).

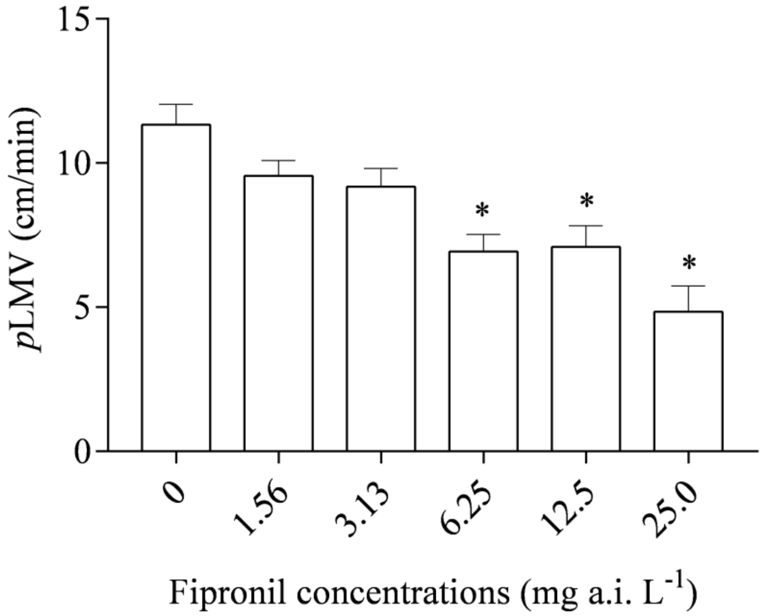

Figure 1: The *p*LMV of the *G. tigrina*, after eight days of exposure to sublethal concentrations to the insecticide based on Fipronil. Data are presented as mean ± standard error. *Significant difference is observed in comparison with the control treatment (Dunnett's post-hoc test).

As a result of the increase in Fipronil concentrations, the exposed planarians suffered a significant delay in complete regeneration ($F_{(5, 84)} = 45.77$; $p < 0.0001$; Fig. 2 C), presenting a LOEC of 1,56 mg L^{-1}, and the auricles of ($F_{(5, 84)} = 87.89$; $p < 0.0001$; Fig. 2 B), compared to control treatment. As photoreceptor regeneration, planarians suffered significant delay with increasing concentrations (H = 77.5; $p < 0.0001$; Fig. 2 A). NOEC was set at 1.56 mg a.i. L^{-1}, and LOEC was set at 3.13 mg a.i. L^{-1} for the regeneration of photoreceptors and auricles.

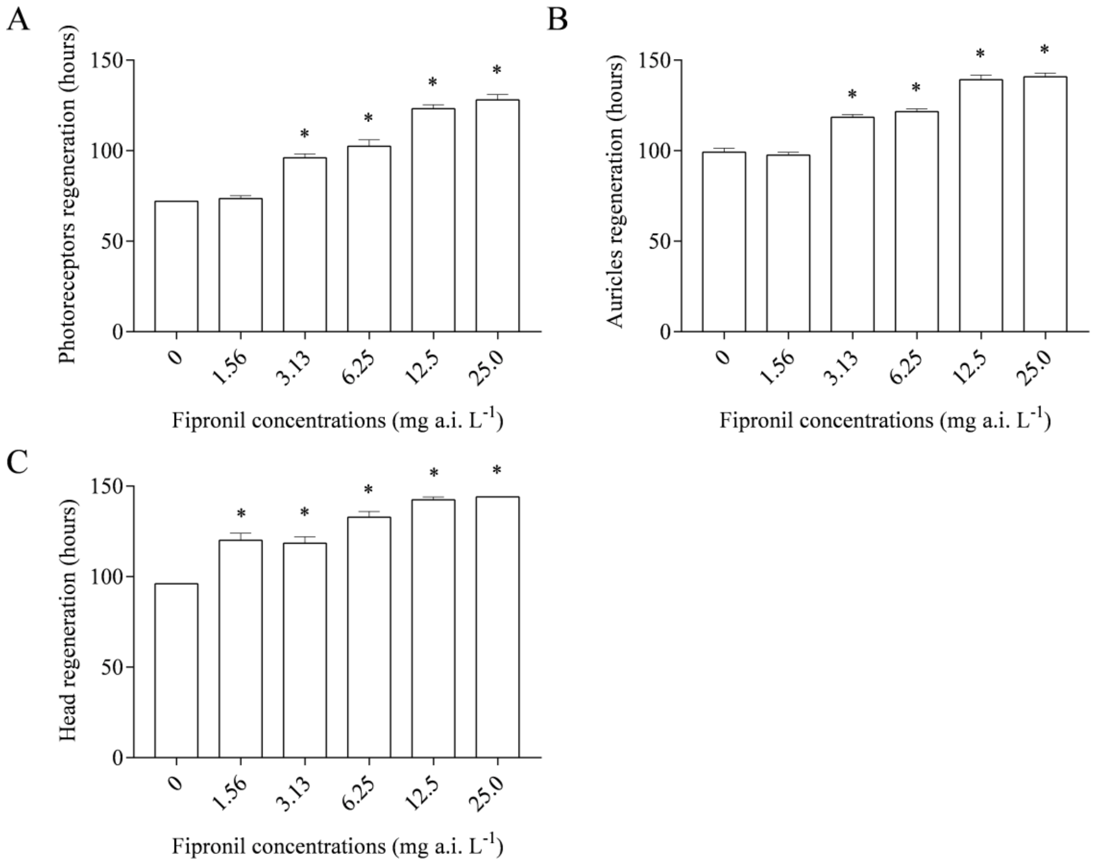

Figure 2: Effects of sublethal concentrations of insecticide based on Fipronil on the regeneration of the *G. tigrina*. A – Photoreceptor regeneration. B – Regeneration of the auricles. C – Complete head regeneration. The exposure time was sixteen days. Data are presented as mean ± standard error. *Significant difference is observed in comparison with the control treatment-(Dunnett's post-hoc test).

The fecundity rate decreased significantly with the increase of the Fipronil concentration ($F_{(5, 10)} = 4.875$; $p < 0.05$; Fig. 3 A). However, concentrations of the pesticide did not, significantly, affect the fertility rate on *G. tigrina* ($F_{(5, 9)} = 1.886$; $p > 0.05$; Fig. 3 B). NOEC was established at the concentration of 6.25 mg a.i. L^{-1} for the fecundity rate, and the LOEC was established at the concentration of 12.5 mg i.a.L^{-1}.

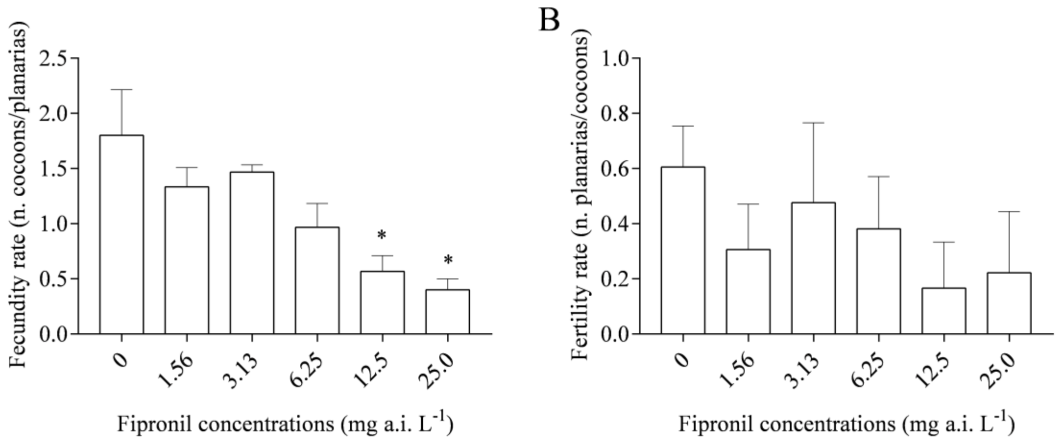

Figure 3: A – Effects of sublethal concentrations of Fipronil on the reproduction of the *G. tigrina*. A – Fecundity rate. B – Fertility rate. Data are presented as mean ± standard error. *Significant difference is observed in comparison with the control treatment (Dunnett's post-hoc test).

4. DISCUSSION

Scientific researches have been developed for the quantification of pesticides with active ingredient based on Fipronil in aquatic ecosystems. Some studies report that the high toxicity of insecticides based on Fipronil contaminate aquatic systems and promote lethal and sublethal effects in non-target aquatic organisms, such as bees, fish, reptiles, aquatic arthropods, and birds (DELSO et al., 2015; PISA et al., 2017). These facts result in an imbalance in these ecosystems (CHAGNON et al., 2015; GIORIO et al., 2017; SUGITA; AGEMORI; GOKA, 2018; HE et al., 2019).

This study reveals that concentrations of the insecticide based on Fipronil up to 1000 mg L^{-1} are not expected to cause mortality of the *G. tigrina*. Nevertheless, *G.* tigrina exhibited less sensitivity to Fipronil when compared to other aquatic organisms, such as *Chironomus riparius* ($CL_{50\text{-}48h}$ = 1.74 µg L^{-1}) (MONTEIRO, et al., 2019), and larvae of *Danio rerio* ($CL_{50\text{-}72h}$ = 13.47 mg L^{-1}) (PARK et al., 2020).

Although lethal toxicity is a short-term effect assessment parameter, sublethal toxicity parameters at the organism level are important for understanding the long-term toxicity of xenobiotics. In this sense, the results of our study showed the behavioral and physiological changes of planarians exposed to the insecticide based on Fipronil. Therefore, this research adds important ecotoxicological data about the sublethal effects of Fipronil in tropical freshwater invertebrates, which may contribute to a more sensitive approach to the analysis of the ecological risk represented using Fipronil or other fungicides of the pyrazole group. In addition, this study highlights the importance of sublethal tests (locomotion, regeneration, and sexual reproduction of planarians) which seems to be much more sensitive than survival bioassays.

The locomotor behavior of the planarians was affected with a LOEC of 6.25 mg a.i. L^{-1}. This negative effect possibly occurred due to the fact that Fipronil affected the nervous system of the planarians. In fact, the locomotor behavior of the planarians is related to the proper functioning of the nervous system, where muscle contraction can be used (NISHIMURA, et al., 2007). Furthermore, the feeding activity of the planarians is directly related to their locomotor capacity for capturing prey, since the planarians are predator/prey organisms, generating an imbalance in the food chain (OFOEGBU et al., 2019; LÓPEZ et al., 2021).

The regeneration of *G. tigrina* was the most sensitive parameter after exposure to Fipronil compared with other evaluated parameters. There was a significant delay in the regeneration of photoreceptors and auricles from the concentration of 3.13 mg a.i. L^{-1}, and the complete head regeneration was significantly delayed from exposure by 1.56 mg a.i. L^{-1} of Fipronil. Thus, this result also shows that Fipronil may have altered the nervous system of planarians or the planarian regeneration act. The process of muscle contraction and the extension of the epidermis in the wound is necessary as a process that leads to the closure of the lesion. Because of these changes, the cells identified as blastema are organized under the epidermis and the replacement of the missing segments is processed (WENEMOSER; REDDIEN, 2010; ORSO et al., 2021).

As a consequence of the delay in the regeneration of photoreceptors, planarians can suffer from changes in the dynamics of their populations, as from influence of the perception of the intensity and direction of light, as well as it´s said that the delay in the regeneration of auricles can limit the perception of the chemical sensations of the environment, including food capture (VILA-FARRÉ; RINK, 2018).

There was also a reduction in the fertility rate of planarians, presenting a LOEC of 12.5 mg a.i. L^{-1}. However, since Fipronil did not, significantly, affect the fertility rate, there was a reduction with fewer numbers of offspring planarians. Fipronil affected the development of the *G. tigrina* as well as other aquatic organisms, for example, such as *Danio rerio* exposed in concentrations from 2.5 mg a.i. L^{-1} that had its development affected.

High toxicity of Fipronil is observed for the insect *C. riparius*, due to the insecticidal action of Fipronil. High toxicity was also expected for planaria; on the other hand, planarians showed similar sensitivity when compared to zebrafish, which is a model organism in ecotoxicology. So this study demonstrates the importance of planarians as candidates for bioindicators of environmental contamination, through ecotoxicological assays.

This scientific research reports that insecticides based on Fipronil cause lethal (survival) and sublethal (behavior and physiology) effects in aquatic and terrestrial organisms. Regarding sublethal effects, the action of this insecticide on non-target organisms, namely bees and even other species of insects, amphibians, reptiles, and birds, can cause damage to the immune systems and to the reproduction of these organisms, and thus, contribute to the decrease of the population of these species (PISA et al., 2017).

Although studies on the impact of Fipronil on non-target organisms have been more notorious in recent years, there are still few studies addressing the lethal and sublethal effects of Fipronil in aquatic invertebrates. However, some studies have already reported the toxicity of Fipronil in other organisms (Table 1). Thus, this study shows that low concentrations of the active ingredient of Fipronil cause chronic effects in planarians (*G. tigrina*), such as delay in locomotion, regeneration, and reproduction (fecundity). It can be concluded that the study will certainly collaborate with the ecological risk analysis of Fipronil in freshwater systems, aiming to protect aquatic ecosystems.

Table 1 – Effect of Fipronil on organisms

Effect of Fipronil on organisms				
Species	**Group**	**Parameters**	**Toxicity (mg L^{-1})**	**Reference**
Chlamydomonas reinhardinhartii	Seaweed	Survival	2.44	PINO-OTÍN et al. (2020)
Chironomus riparius	Insects	Survival	0.00084	MONTEIRO et al. (2019)
Chironomus sancticaroli	Insects	Survival	0.0037	PINTO et al. (2021)
Cyprinus carpio	Fish	Biochemicals	0.65	CLASEN et al. (2012)
Danio rerio	Fish	Behavior	0.0002 to 2.00	EADIE et al. (2020)
Daphnia magna	Crustacean	Survival	0.07 to 0.38	PINO-OTÍN et al. (2020)

Partamona helleri	Insects	LC_{50}	0.00000028	FARDER-GOMES et al. (2021)
Silurana tropicalis	Reptiles	Survival	1.34 to 3	SAKA; TADA (2021)
Spodoptera litura	Insects	Molecular, biochemical and organic	20 to 80	JAMEEL et al. (2019)

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CHAPTER II

Environmentally relevant concentrations of chlorpyrifos affect the behavioral and physiological parameters of *Girardia tigrina*

ABSTRACT

Chlorpyrifos is a toxic organophosphate insecticide, which is widely used in the control of insect pests in agriculture, such as soybeans, corn, coffee, sorghum, wheat, beans, and barley. Thereby, Chlorpyrifos has been reported to reach aquatic systems, so the potential of acute and chronic effects of this insecticide on non-target organisms are alarming (this is because insects, even if non-target ones, are the most expected for an insecticide to cause toxicity). Thus, the present study aimed to conduct studies of laboratory ecotoxicological tests with tropical freshwater planarians, for toxicity, assessment, through acute (survival) and chronic (regeneration, locomotion, and reproduction) ecotoxicological tests bioassays. The results of an acute toxicity process/ test revealed CL_{50} of Chlorpyrifos over planarians equal to 622.8 µg a.i L^{-1}. Additionally, the chronic toxicity test for effects on the locomotion of *G. tigrina* revealed NOEC - No Observed Effect Concentration of 7.75 µg a.i. L^{-1} and LOEC - Lowest Observed Effect Concentration of 15.5 µg a.i. L^{-1}. On the other hand, for regeneration tests, LOEC was already established at the lowest study concentration, 3.88 µg a.i. L^{-1}. From the results presented, high toxicity of Chlorpyrifos to the planaria *G. tigrina* is observed, in environmentally relevant concentrations 37.3 µg L^{-1} while our study demonstrates the potential of planaria to bioindicated contamination in tropical freshwater ecosystems by Chlorpyrifos. Finally, based on the results of acute and chronic toxicity in planaria, at the organism level, this study may contribute to other studies that evaluate the toxicity of this insecticide on other aquatic organisms, including at higher levels of biological organization, such as populations of aquatic organisms.

Keywords: Acute effect, Chronic effect, Ecotoxicology, Non-target aquatic organisms, Organophosphate insecticide

1. INTRODUCTION

Areas of intensive agricultural production have been reported to contribute considerably to the contamination of aquatic ecosystems, mainly due to the intensive use of pesticides (BARBOSA; SOLANO; UMBUZEIRO, 2015). Generally, intensive monoculture areas occur mainly closed to water resources and, in this context, several studies on the occurrence and quantification of active ingredients of pesticides in surface waters have been reported in the literature (PAPADAKIS et al., 2018; KNILLMANN et al., 2018; MAMUN et al., 2019; ROBINSON et al., 2019; TSABOULA et al., 2019).

Among the wide diversity of pesticides, the active ingredient (i.a.) Chlorpyrifos (*O,O-diethyl O-3,5,6-trichloropyridin-2-pyridinyl phosphorothioate*) is an insecticide that acts on the nervous system of insects and it is widely used in agriculture to control insect pests (GIESY et al., 2014). Chlorpyrifos has been reported as a potential insecticide to reach water resources, mainly by runoff, whereas a recent species sensitivity distribution analysis study reports the Chlorpyriphos toxicity for 207 species of different aquatic organisms including vertebrates and invertebrates (ZHAO; CHEN, 2016).

Chlorpyrifos has low water solubility; it is not mobile, and it is highly volatile. Based on its chemical properties, it has low leaching potential for groundwater, thus it is not persistent in water, however, it is moderately persistent in the soil, as well as highly toxic to mammals, birds, fishes, aquatic invertebrates, and bees, and moderately toxic to aquatic plants, algae, and earthworms (PPDB, 2021). Chlorpyrifos can reach freshwater ecosystems, and the environmentally expected concentration in surface water is 37.3 μg/L (HASANUZZAMAN et al., 2018).

In ecotoxicology, freshwater planarians *Girardia tigrina* (Girard, 1850) (Paludícola: Dugesiidae), are aquatic organisms reported as bioindicators of environmental contamination, due to their physiological and behavioral characteristics (DORNELAS et al., 2020; MACÊDO et al., 2019). Planarians are candidates for good test organisms, as they are predators, as well as prey, and for the ease of monitoring in the laboratory (LÓPEZ, et al. 2019). In addition, research at international level in several areas of science, such as pharmacology and ecotoxicology, has used planarians in laboratory tests, in consequence of the tests which require less time and are easier, when compared with the tests using other organisms (SHIROOR; BOHR; ADLER, 2020;

TASHIRO et al., 2014; WU; LI, 2018). Thereby, the objective of this study is to evaluate the acute and chronic toxicity of insecticide based on Chlorpyrifos on planarians.

2. MATERIAL AND METHODS

2.1 Test organisms

Planarians *G. tigrina* culture were kept at Plant Metabolism and Ecotoxicology Laboratory of IF Goiano – Campus Rio Verde, in American Standard Test and Materials aqueous medium (ASTM) (ASTM, 1980), under a constant temperature of 22 ± 1 °C, in absence of light. Once a week these organisms were fed, *ad libitum*, with bovine liver, and the medium was renewed posteriorly. One week before testing, the organisms were not fed to prevent contamination of the experiment by digesting food and to ensure uniformity in toxicity response. (OVIEDO; NICOLAS; ADAMS, 2015).

2.2 Chlorpyrifos insecticide

Chlorpyrifos was prepared from a commercial formulation liquid (Ameri bras®), with 480 g L^{-1} of active ingredient concentration. A stock solution of 100000 µg a.i. L^{-1} was prepared in distilled water. The stock solution was protected from light and stored at a temperature of 4 °C to prevent degradation. Experimental solutions were prepared by diluting the stock solution in ASTM medium.

2.3 Acute effect on *G. tigrina*

The lethal concentration (CL_{50}) for *G. tigrina* was determined after 48 hours of exposure to Chlorpyrifos, and compare to the control treatment (ASTM medium). Eight nominal concentrations (280, 340, 410, 490, 580, 700, 840 and 1000 µg L^{-1}) were used. The tests were carried out in polyethylene terephthalate (PET) flasks containing 20 mL of experimental solution at 22 ± 1 ° C. Five replicates were prepared for each concentration and each flask contained four planarians (1.0 ± 0.2 cm full-length). All test dilutions were prepared using ASTM medium (adapted from SARAIVA et al., 2018; LÓPEZ et al., 2019).

2.4 Chronic effect on *G. tigrina* for determination of NOEC and LOEC

In the experiments, in order to evaluate the locomotion and regeneration process, planarians of 1.0 ± 0.2 cm full-length were used, being exposed to different concentrations of Chlorpyrifos for eight days, with the following nominal concentrations: 3.88, 7.75, 15, 31, and 62 µg L^{-1}. The tests were conducted under dark conditions at 22 ± 1 °C, in which the planarians were deprived of food one week before the beginning of the test and during the experimental test. The exposure was carried out with a group of 30 organisms divided into three replicates per treatment, in glass beakers, containing 100 mL of experimental solution. Within four days, the test solutions with the respective concentrations were renewed. The control treatment contained only ASTM medium, under the same conditions as the other experimental treatments. Then, to evaluate the effects on locomotion and regeneration with the respective concentrations, after eight days of exposure, the planarians were introduced in new test solutions (adapted from SARAIVA et al., 2018; LÓPEZ et al., 2019).

2.4.1 Planarian locomotor velocity (*p*LmV)

To evaluate planarian locomotor velocity (*p*LmV) a round container covered with a lined sheet of paper was used (lines spaced at 0.5 cm) and ASTM medium was used for covering the bottom. After thirty seconds of adaptation, the procedure followed by observation of the centimeters traveled by the planarians in a period of two minutes (adapted from SARAIVA et al., 2018; LÓPEZ et al., 2019; PESTANA; OFOEGBU, 2021).

2.4.2 Regeneration

Fifteen planarians were selected from each concentration and they were decapitated, with a single cut behind the auricles. After decapitation, the planarians were transferred to a PET flask with 20 mL of experimental solution. All process of regeneration including the formation of photoreceptors, auricles, and complete head formation was evaluated with a Magnifier Lamp, monitoring the number of hours until the formation of new photoreceptors and auricles, as well as the entire head (adapted from SARAIVA et al., 2018; LÓPEZ et al., 2019; PESTANA; OFOEGBU, 2021).

2.4.3 Reproduction

For the reproduction test, the organisms were exposed to insecticide for 4 weeks to assess fertility and for 3 weeks to assess fecundity. The planarians were used at the beginning of the reproductive age (1.5 ± 0.1 cm in length). Five different nominal concentrations were used, as described for the previous parameters, with 3 replicates per treatment, with each repetition containing 10 organisms. These organisms were introduced in PET flasks, containing 100 mL of experimental solution. Weekly, the solutions of each concentration were replaced by new solutions, after feeding the organisms with bovine liver (*ad libitum*). The experiment was carried out at 22 ± 1 ° C, in the absence of light and it was observed daily. Fecundity was evaluated by the number of cocoons produced per day, divided by the number of exposed planarians. Nevertheless, the fertility was determined by the number of offspring (planarians that were born from the cocoons), divided by the number of deposited cocoons (adapted from DORNELAS et al., 2020; KNAKIEVICZ et al., 2006).

2.5 Statistical analysis

The acute toxicity (CL_{50}) of Chlorpyrifos in *G. tigrina* was estimated by using a dose-response curve. The chronic toxicity parameters of the exposure of the planarians to Chlorpyrifos were evaluated by analysis of variance (ANOVA) and, successively, the Dunnett's *post-hoc* test was applied to assess significant differences between treatments. To verify whether the data agreed with ANOVA assumptions, locomotion and reproduction tests were analyzed for homogeneity of variances and normality, by using the Bartlett and Kolmogorov-Smirnov tests, respectively. The regeneration tests were not in accordance with the assumptions of ANOVA analysis, therefore, it was necessary to use nonparametric statistics, using the Kruskal Wallis test (Dunns' *post-hoc* test).

Statistical analysis was performed by using the GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, California. EUA).

3. RESULTS

3.1 Acute effect of Chlorpyrifos on planarians

The lethal effect (CL_{50} 48h– 95% IC) of Chlorpyrifos for *G. tigrina* was 622.8 µg i.a. L^{-1} (minimum 582 and maximum 664.8 µg i.a. L^{-1}). At the end of the exposure, no mortality was observed in the control treatment.

3.2 Chronic effects of Chlorpyrifos in planarians

Locomotion speed (*p*LMV) of *G. tigrina* decreased significantly after exposure to Chlorpyrifos when compared to ~~the control treatment~~ ($F_{(5, 84)} = 28.29$; $p < 0.0001$). It was showed LOEC (Lowest Observed Effect Concentration) of the 3.88 µg i.a.L^{-1} (Fig. 1).

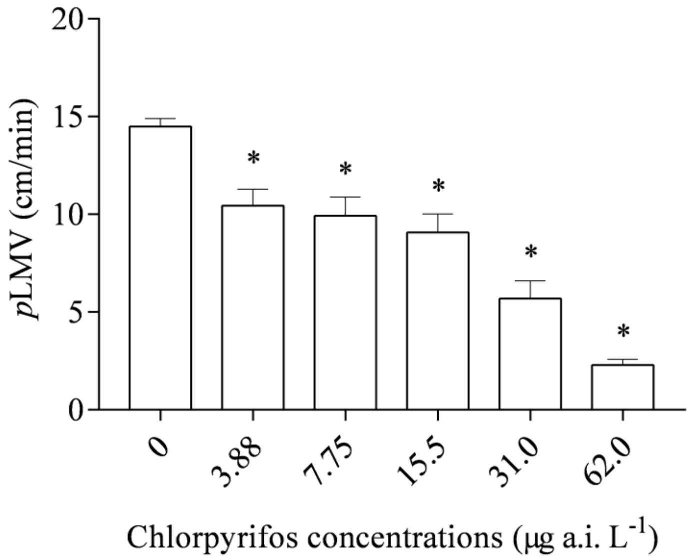

Figure 1: The *p*LMV of *G. tigrina*, after eight days of exposure to sublethal concentrations of Chlorpyrifos. Data are presented as mean ± standard error.*Significant difference is observed in comparison with the control treatment (Dunnett's *post-hoc* test).

Regeneration was measured every 24 hours until complete head formation (Fig. 2 C), photoreceptors (Fig. 2 A), and auricles (Fig. 2 B). According to the increase in concentrations, the exposed planarians suffered a significant delay in complete regeneration ($F_{(5, 84)} = 28.5$; $p < 0.0001$; Fig. 2 C), photoreceptors (H= 37.49; $p < 0.0001$; Fig. 2 A) and auricles ($F_{(5, 84)} = 3.663$; $p > 0.05$; Fig. 2 B), compared to control treatment. For evaluation of the complete head regeneration, the LOEC was set at 3.88 µg i.a. L^{-1}. For photoreceptor and auricles regeneration, the NOEC was 15.5 µg i.a. L^{-1} and the LOEC was 31.0 µg i.a. L^{-1}. Also, it was observed that in one of the replicas an organism suffered injuries, in the concentration 62 µg i.a. L^{-1}.

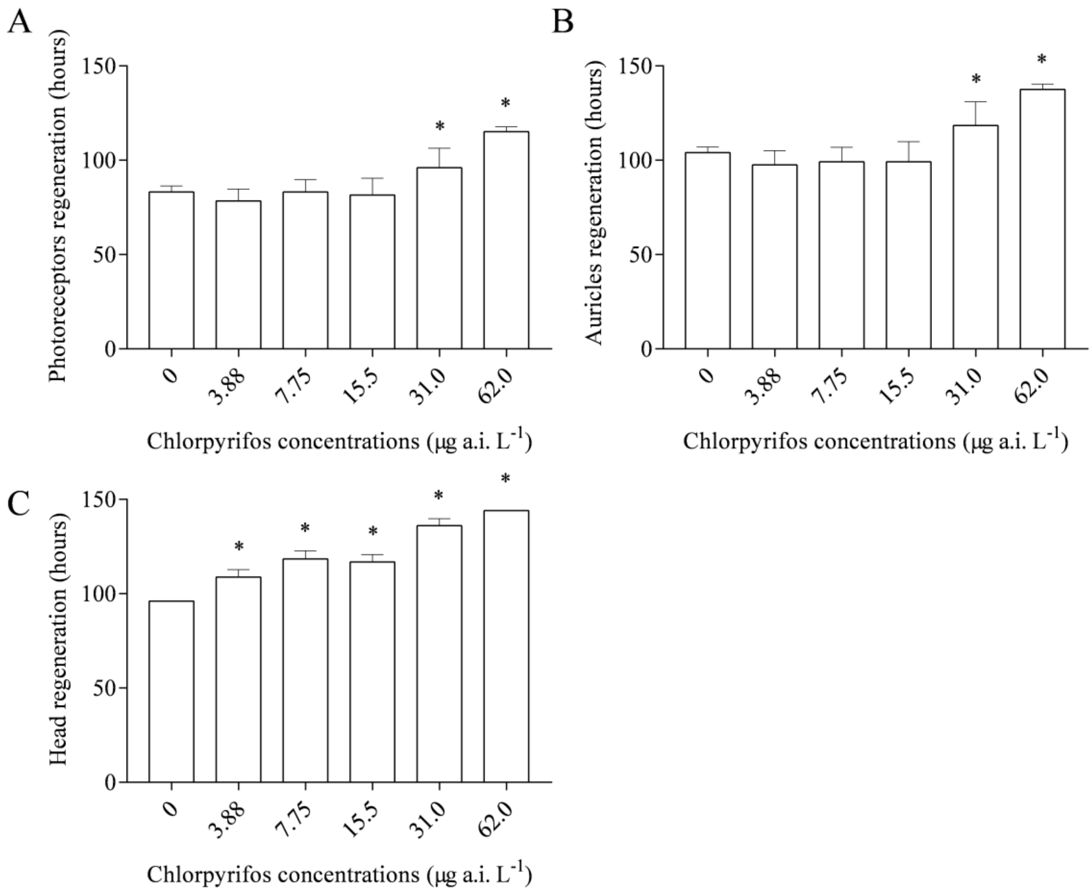

Figure 2: Effects of sublethal concentrations of Chlorpyrifos on the regeneration of *G. tigrina*. A – Photoreceptor regeneration. B – Regeneration of the auricles C - Complete head regeneration. Data are presented as mean ± standard error. *Significant difference is observed in comparison with the control treatment (Dunns' *post-hoc* test).

Planarian reproduction was evaluated in terms of fecundity rate and fertility rate. No significant reduction in fecundity rates ($F_{(5, 12)} = 0.514$; $p > 0.05$; Fig. 3 A) or in fertility rate ($F_{(5, 84)} = 3.979$; $p < 0.05$; Fig. 3 B) were observed on planarians. Lesions were observed in planarians exposed to 62 µg a.i. L^{-1}. (Fig. 3).

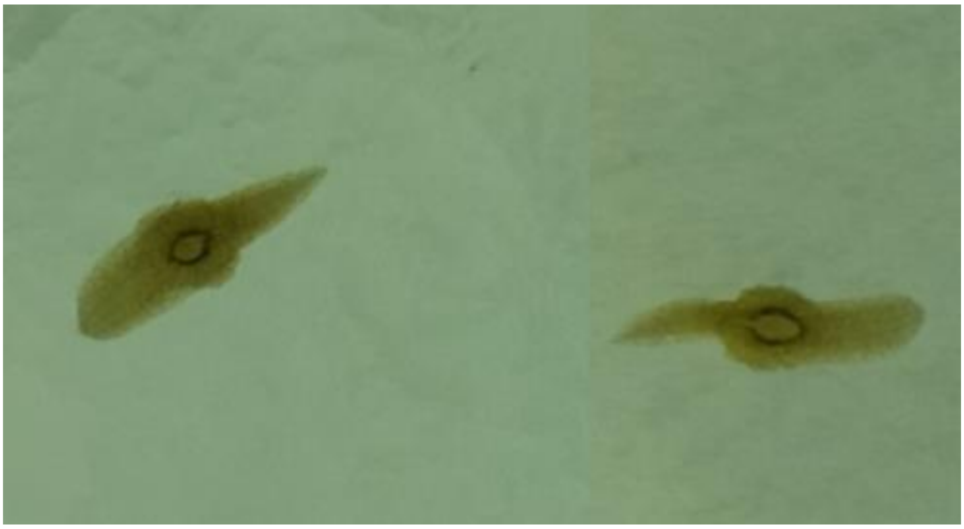

Figure 3 - Injury caused in planarians (Chlorpyrifos concentration 62 µg a.i. L^{-1}) exposed for a period of 15 days.

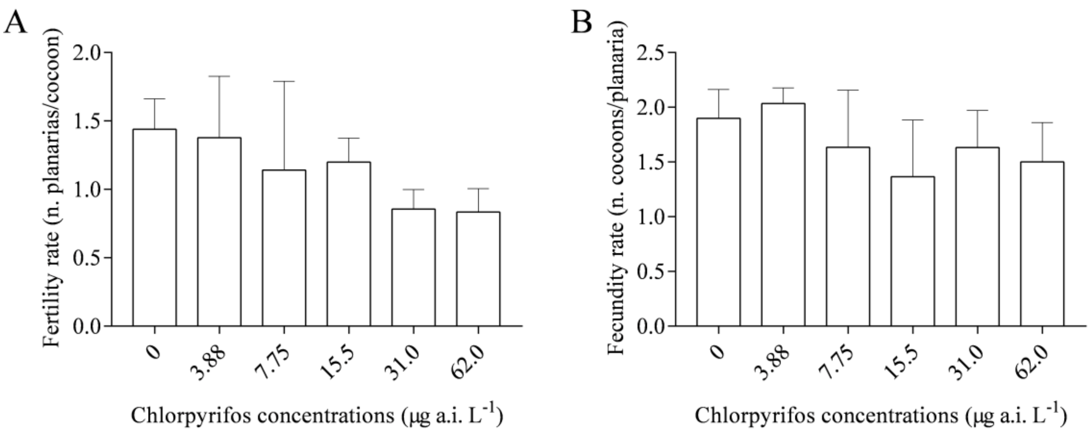

Figure 4: Effects of sublethal concentrations of Chlorpyrifos on the reproduction of *G. tigrina*. A – Fecundity rate. B - Fertility rate. Data are presented as mean ± standard error.

4. DISCUSSION

This study provides relevant information on the negative effects of Chlorpyrifos on non-target tropical freshwater aquatic invertebrates *G. tigrina*, i.e., by affecting the individual responses of the organisms (decreasing locomotor activity and delayed regeneration), in addition to survival, with potential deleterious impact on the planarians population. This knowledge about the collateral effects caused by Chlorpyrifos in non-target aquatic organisms is of fundamental importance for the assessment of impacts and risks in freshwater ecosystems.

The literature reports the acute and chronic toxicity of Chlorpyrifos on aquatic organisms, however, there are still no studies with planarians. Thus, this research highlights the relevance of using planarians in laboratory tests, as these organisms are easy to maintain in the laboratory, have a low economic cost, are effective in tests, and, in addition, present biological functions which are similar to other organisms.

The insecticide Chlorpyrifos was toxic to the invertebrate *G. tigrina* (planarian), after exposure for 48 h, presenting a CL_{50} of 622.8 µg a.i. L^{-1}. Thereby, planarians showed greater sensitivity to the insecticide Chlorpyrifos, compared to fish *Anguilla anguilla* (CL_{50} = 783.329 µg a.i. L^{-1}), to insect *Anopheles sinensis* (CL_{50} = 4700 µg a.i. L^{-1}), to mollusk *Bulinus truncatus* (CL_{50} = 1320 µg a.i. L^{-1}), to algae *Chlorella sp* (CL_{50} = 1290 µg a.i. L^{-1}) and to crustacean *Neocaridina denticulata* (CL_{50} = 692.913 µg a.i. L^{-1}) (ZHAO; CHEN, 2016).

According to the results of the sublethal tests, with exposure to Chlorpyrifos concentrations of 3.88 and 31.0 µg a.i. L^{-1}, there was a reduction in locomotor speed and a delay in the regeneration of the planarians. The limitation in locomotion may be related to changes in body surface and neurotoxicity related to exposure to chemical agents, due to the fact that the planarians move by sliding and this activity is totally muscular (OVIEDO et al., 2008; TALBOT; SCHOTZ, 2011). The reduction in the locomotion of planarians can also imply a greater vulnerability to attack by predators since these organisms are predators and prey (ROFRIGUES et al., 2016; MACÊDO et al., 2019).

The delay in the regeneration of the planarians' head causes negative effects for the organism. Indeed, planarians detect the presence of prey nearby and find food using their photoreceptors, and they ingest food by using their retractable pharynx for ingestion. Once these organisms are negatively phototropic, the delay in photoreceptor regeneration affects the detection of light intensity with possible consequences in terms of preventing predators or foraging (KOLASA, 2001).

Thus, the absence of these organisms in the aquatic ecosystem causes changes in the entire food chain (RODRIGUES et al., 2016). On the other hand, the delay in locomotion and head regeneration turn these organisms more vulnerable to predator actions (OFOEGBU et al., 2016).

Planarians proved to be more sensitive to the insecticide Chlorpyrifos than fish (*Pseudetroplus maculatus*) when exposed to concentrations of 0.661 µg a.i. L^{-1} and 1.32 µg a.i. L^{-1} showing changes in immunological, hormonal, and histological (RAIBEEMOL; CHITRA, 2020). Moreover, they proved to be more sensitive than tadpoles (*Rhinella arenarum*), and when exposed to Chlorpyrifos they had a decrease of locomotor performance in approximately 50% at the maximum concentration of Chlorpyrifos (75 µg i.a. L^{-1}) (QUIROGA, et al., 2019).

Finally, based on chronic toxicity data, this study reports the effect of Chlorpyrifos in *G. tigrina* at predicted concentrations to reach aquatic environments, i.e., 37.3 µg i.a. L^{-1} (HASANUZZAMAN et al., 2018), which reinforces the importance of biomonitoring of this insecticide in natural aquatic systems. It demonstrates the potential use of planarians as a bioindicator of environmental contamination by Chlorpyrifos. Moreover, the chronic effect observed at concentrations as low as those found in the aquatic environment shows the importance of this study for contributing to the ecological risk analysis of Chlorpyrifos in freshwater ecosystems.

It is known that the intensive and periodic use of insecticides can potentially impact aquatic ecosystems. Thus, the ecotoxicological approach through laboratory studies of this research, turned to be the basis for future studies of species sensitivity analysis, in addition, by contributing to environmental protection agencies, within the scope of ecological risk analysis and toxicological reassessment of insecticides a Chlorpyrifos base. The main results obtained through the study revealed that low concentrations of insecticide based on Chlorpyrifos caused lethal and sublethal effects (effects observed at environmentally relevant concentrations) in *G. tigrina*.

The scientific research using planarians for toxicological evaluation of chlorpyrifos is scarce in the scientific literature. However, there is a range of studies with other organisms (Table 1) to assess the lethal and sublethal toxicity of Chlorpyrifos, assessing their effects at the physiological, morphological, biochemical level as well as the survival.

Table 1 – Effect of Chlorpyrifos on organisms

Effect of Chlorpyrifos on organisms				
Species	**Group**	**Parameters**	**Toxicity (mg L^{-1})**	**Reference**
Acartia tonsa	Copepods	LC_{50}	0.00134	BELLAS; GIL (2020)
Acartia tonsa	Copepods	EC_{50}	0.00077 - 0.001047	BELLAS; GIL (2020)
Cyprinus carpio	Fish	LC_{50}	0.440	KUNWAR et al. (2021)
Danio rerio	Fish	LC_{50}	1.35	QIAO et al. (2021)
Danio rerio	Fish	Acetylcholinesterase inhibitor	0.013	QIAO et al. (2021)

Eisenia fetida	Worms	Subchronic	0.01 - 1.00	ZHU et al. (2020)
Litopenaeus vannamei	Shrimp	LC_{50}	0.00068	PAWAR et al. (2020)
Tor putitora	Fish	LC_{50}	0.753	KUNWAR et al. (2021)

This research is of great importance, since it starts to report the use of planarians, namely the species *G. tigrina* as candidates for alternative bioindicator organisms of environmental contamination by Chlorpyrifos. Additionally, this fact is in accordance with the environmental research worldwide, once several countries of the world have encouraged the use of invertebrates for toxicity tests, as well as the use of fast laboratory tests.

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CONCLUSÃO GERAL

Sabe-se que o uso intensivo e periódico de inseticidas podem, potencialmente, impactar ecossistemas aquáticos. Desse modo, ao fazer-se uso da abordagem ecotoxicológica através de estudos laboratoriais, os dados da presente pesquisa, uma vez publicados no meio científico internacional, tornaram-se base para futuros estudos de análise de sensibilidade de espécies, além de contribuir com agências de proteção ambiental, no âmbito da análise de risco ecológico e reavaliação toxicológica de inseticidas a base de Fipronil e Clorpirifós. Os principais resultados obtidos revelaram que concentrações de inseticida a base de Fipronil ocasionam efeitos crônicos, como atraso na locomoção, regeneração e reprodução de *G. tigrina*. Do mesmo modo, baixas concentrações de inseticida a base de Clorpirifós, provocaram efeitos agudo e crônicos (efeitos observados à concentrações ambientalmente relevantes) em *G. tigrina*.

Esta pesquisa passa a reportar o uso de planárias, nomeadamente a espécie *G. tigrina*, como bons organismos bioindicadores de contaminação ambiental por Fipronil e Clorpirifós, e vai ao encontro do que tem prezado a ciência ambiental, que em diversos países do mundo tem incentivado o uso de invertebrados para ensaios de toxicidade, bem como ensaios laboratoriais rápidos e a baixo custo.

Como estudos futuros, tornam-se interessantes e potenciais, pesquisas laboratoriais que avaliem planárias como bioindicadores da contaminação de águas superficiais, coletadas em ambientes aquáticos, próximos a áreas de produção agropecuária intensiva. Assim, torna-se oportuno não somente a quantificação de contaminantes num determinado sistema aquático, mas, também, uma indicação biológica dos efeitos potenciais dos xenobióticos presentes nas águas superficiais.