UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS
RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
BIOLOGIA CELULAR E MOLECULAR

POTENCIAL TÓXICO E GENOTÓXICO DO INSETICIDA IMIDACLOPRIDO EM ORGANISMOS NÃO ALVOS

YADIRA ANSOAR RODRÍGUEZ

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular)

Julho - 2016

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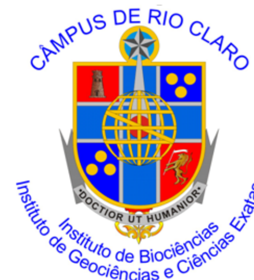

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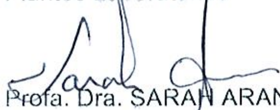

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“Sou livre para o silêncio das formas e das cores''

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Perfecto distingo lo negro del blanco…

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Los dos materiales que forman mi canto

Y el canto de ustedes que es el mismo canto

Y el canto de todos que es mi propio canto.

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RESUMO

A aplicação indiscriminada de agrotóxicos constitui uma das maiores preocupações na atualidade, sendo o Brasil um dos países que mais uso faz destes produtos. O imidacloprido (IMI) é um dos inseticidas mais utilizados no mundo, principalmente nas culturas de cana-de-açúcar, citros, algodão e café. Apesar de seus benefícios, pode apresentar potencial tóxico e genotóxico em organismos não alvo. O uso de bioindicadores permite o estudo dos possíveis riscos destas substâncias nos organismos. Entre estes, plantas superiores e organismos aquáticos são considerados excelentes para avaliar efeitos de agrotóxicos no ambiente. Neste estudo, foram avaliados os efeitos de IMI em organismos não alvos (*Allium cepa*, *Tradescantia pallida* e *Oreochromis niloticus*) expostos a diferentes concentrações, baseadas na aplicação deste inseticida na cultura de cana-de-açúcar, por meio de ensaios celulares e moleculares. Foram testadas concentrações equivalentes à dose do produto recomendada para esta cultura (400 g/ha), a metade (200 g/ha) para simulação da diluição natural e o dobro (800 g/ha) para simulação do uso indiscriminado. O teste de aberrações cromossômicas e de micronúcleos (MN) em *A. cepa* e *T. pallida* foram utilizados para avaliar a toxicidade e genotoxicidade. Ensaio do cometa e teste do MN em eritrócitos de *O. niloticus*, avaliaram danos em nível primário e cromossômico. Análise das alterações histopatológicas no fígado de *O. niloticus* e localização *in situ* das proteínas de choque térmico (HSP70) analisadas sob microscopia de luz e imuno-histoquímica, respectivamente, foram empregadas para verificar o potencial tóxico em nível celular. Os resultados no teste de *A. cepa* e *T. pallida*, demonstraram que o IMI induziu alterações cromossômicas e o aumento da frequência de MN. Também foi observado indução do dano primário em eritrócitos de *O. niloticus* nas concentrações testadas e dano em nível cromossômico na maior concentração. Alterações hepáticas também foram observadas em todas as concentrações testadas, entre elas: degeneração hidrópica, núcleos picnóticos e perda do limite celular. A concentração mais alta (250µg/L) induziu um aumento de lípidos ácidos e neutros e dos níveis de marcação da proteína HSP70. Diante dos resultados pode-se concluir que o IMI, nas concentrações testadas, foi genotóxico para os organismos, além de induzir alterações histopatológicas e ativar mecanismos citoprotetores mediados por proteína de choque térmico. Este inseticida apresentou potencial tóxico e genotóxico nos organismos testados, os quais não são alvos de ação deste agrotóxico, fato este que deve ser levado em consideração para sua aplicação.

Palavras-chave: *Allium cepa*, *Tradescantia pallida*, *Oreochromis niloticus,* agrotóxicos, micronúcleos, alterações cromossômicas, ensaio do cometa, histopatologia, HSP70.

ABSTRACT

The indiscriminate application of pesticides is a major concern nowadays, and Brazil is one of the countries which use these products heavily on agriculture. The imidacloprid (IMI) is an insecticide sold worldwide, being widely used on sugar cane, citrus, cotton and coffee crops. Despite its benefits, IMI may have potential for inducing genetic changes in non-target organisms. In this sense, the use of bioindicators like higher plants and fishes allow the assessment of possible effects and risks to the environment derived from the use of this insecticide in agriculture. In this study, we evaluated the effects of IMI on non-target organisms (*Allium cepa, Tradescantia pallida* and *Oreochromis niloticus*) exposed to different concentrations, based on the application of this insecticide in the sugarcane culture, through cellular and molecular assays. A concentration equivalent to the recommended dose of the product for this culture (400 g/ha), the half (200 g/ha) for simulation of natural dilution and double (800 g/ha) that simulates the indiscriminate use. Chromosomal aberrations and micronucleus test (MN) in *A. cepa* and *T. pallida* were analyzed for toxicity and genotoxicity study. Comet assay and MN test in *O. niloticus* erythrocytes assess damage to primary and chromosomal level. Analysis of histopathological changes in the *O. niloticus* liver and *in situ* localization of heat shock protein (HSP70) were analyzed by light microscopy and immunohistochemistry, respectively, were performed to measure the toxic potential at the cellular level. The results in *A. cepa* and *T. pallida* assay, demonstrated that the IMI induced chromosomal alterations and increased frequency of MN. It was also observed induction of primary damage in *O. niloticus* erythrocytes at all concentrations and damage to the chromosomal level in the highest concentration. Liver changes were also found in all tested concentrations, as: hydropic degeneration, pyknotic nuclei and loss of cell limits. The highest concentration (250µg/L) showed an increase of acid and neutral lipids and labelling levels of HSP70 protein. Given the results it is possible to concluded that the IMI in the tested concentrations was genotoxic in these organisms, besides inducing histopathologic changes and activated cytoprotective mechanisms mediated by heat shock protein. This insecticide has a toxic and genotoxic potential for these organisms, which are not the target of action of this pesticide; fact should be considered for its application.

Key-words: *Allium cepa*, *Tradescantia pallida*, *Oreochromis niloticus,* pesticides, micronucleus, chromosome aberrations, comet assay, histopathological, HSP70.

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1. INTRODUÇÃO E RELEVÂNCIA DO TEMA

As reservas de combustível fóssil, base da obtenção mundial de energia, estão sendo esgotadas. Na pesquisa de novas fontes de energias, surgiram os biocombustíveis como potencial substituto do combustível fóssil, com algumas vantagens descritas como, por exemplo, seu potencial para reduzir em até 80% a emissão de gases relacionados ao efeito estufa (MOFIJUR et al., 2016). Entre os principais biocombustíveis encontra-se o bioetanol, que pode ser produzido a partir de plantas propositalmente cultivadas, tais como cana-de-açúcar, beterraba, milho, trigo, sorgo, cevada e outros tipos de biomassas (MASUM et al., 2013).

Dentre os países com significativa produção de biomassa está o Brasil, como o maior produtor de cana-de-açúcar do mundo, destinando 50% de sua produção para a obtenção de bioetanol, com aproximadamente 97-98% desta produção utilizada no setor do transporte (SU et al., 2015). De acordo com a União da Indústria da Cana-de-Açúcar (UNICA, 2015) a safra de 2013/2014 foi de 653.519 toneladas de açúcar e 27.543 m³ de etanol, sendo o estado de São Paulo o responsável por 60% da produção do país, com uma safra de 367.450 toneladas de açúcar e 13.944 m³ de etanol (UNICA, 2015). Entretanto, para poder alcançar a meta de biocombustíveis para o ano 2021, várias simulações realizadas predizem que a cana precisará se expandir sobre uma área entre 5,7 e 6,4 milhões de hectares (LAPOLA et al., 2010; (GOLDEMBERG et al., 2014).

Preocupações de cunho social e ambiental, associados à produção de etanol, em longo prazo, levaram à criação de indicadores de sustentabilidade ambiental para garantir que os custos não superem os benefícios para a sociedade e o meio ambiente (EFROYMSON et al., 2013; EGESKOG et al., 2014). Entretanto, devido à essa complexa relação, muitas questões têm sido levantadas, principalmente pelos efeitos negativos observados, como a perda de habitas e de espécies de flora e fauna, degradação dos solos, expansão dos cultivos na selva tropical, poluição atmosférica, contaminação das águas e incremento no consumo de agrotóxicos (TSAO et al., 2012; PALMER; OWENS, 2015).

A utilização de agrotóxicos, no Brasil, foi incrementanda com o aumento das culturas para a obtenção de biocombustíveis e o uso de sementes transgênicas. O país lidera hoje o ranking de consumo mundial de agrotóxicos com mais de um milhão de toneladas, o que equivale a um consumo médio de 5,2 kg de veneno agrícola por habitante (INCA, 2015). Hoje, o aumento das áreas agrícolas destinadas às culturas de cana-de-açúcar junto com o incremento

no consumo de agrotóxicos e sua aplicação indiscriminada, constitui uma das maiores preocupações atuais.

A utilização de tais insumos na agricultura iniciou-se na década de 1920, época em que eram pouco conhecidos do ponto de vista toxicológico. Dentre esses insumos, a classe dos inseticidas neonicotinóides é uma das mais utilizadas no Brasil e no mundo, com registros em mais de 120 países (GOULSON, 2013), sendo o imidacloprido (IMI) o de maior sucesso. Ele foi registrado pela "United States Environmental Protection Agency" (USEPA), no ano de 1994; é um dos inseticidas amplamente utilizados no mundo (DIJK et al., 2013) para o controle de pragas em diferentes culturas e nos mercados de sanidade animal (SUN; LIU, 2016). Pode ser aplicado diretamente nas sementes e/ou no solo, ou ainda nas folhagens por aplicação aérea, a qual tem sido associada com a morte de abelhas em diferentes regiões do Brasil, sendo proibido durante a etapa de floração devido aos seus efeitos negativos (IBAMA, 2015).

A transferência de moléculas de agrotóxicos dos ecossistemas terrestres aos aquáticos é uma preocupação constante na comunidade científica, sobretudo em áreas agrícolas, as quais têm um maior escoamento superficial e irrigação. O IMI pode ser encontrado em vários ambientes: no solo, em que sua persistência é altamente variável e, em corpos de água, onde pode chegar até 80% (KURWADKAR et al., 2013). Devido a sua presença nestes ambientes, pode provocar efeitos tóxicos em plantas e animais, sendo um risco toxicológico para organismos não alvos, tanto terrestres como aquáticos. Estudos que mostram a toxicidade do IMI foram realizados em diferentes organismos como, por exemplo, minhocas (DITTBRENNER et al., 2011), anfíbios, microalgas (MALEY et al., 2012) e abelhas (SOARES et al., 2015; BRANDT et al., 2016; COSTA et al., 2016). Neste contexto, estudos de genotoxicidade que avaliam o potencial do IMI sobre o material genético de diferentes organismos não alvos, são muito importantes. Para tanto, é necessário contar com ferramentas apropriadas para estes estudos. O emprego de organismos vivos (bioindicadores) oferecem uma resposta biológica no nível de individuo, população, comunidade ou ecossistema (ADAMS et al., 2001), capazes de apontar/indicar de alguma forma a presença de estresses gerados pelos poluentes ambientais, é uma forma de monitorar os efeitos prejudiciais dos agrotóxicos no ambiente. Nos bioindicadores podem ser avaliados biomarcadores em diferentes níveis de organização biológica (molecular, bioquímica, celular, fisiológica) (RICKETTS et al., 2004) e classificados em marcadores de exposição, de efeito e suscetibilidade (WHO 2001).

As plantas superiores são excelentes bioindicadores utilizados mundialmente para o monitoramento ambiental (KOLON et al., 2015; HARGUINTEGUY et al., 2016). Tem-se acumulado uma ampla bibliografia de ensaios de genotoxicidade em plantas como *Allium cepa* (ANDRIOLI et al., 2012; PRATTE-SANTOS et al., 2015). Esta planta é utilizada para avaliar danos no DNA, observados por meio da análise de aberrações cromossômicas e distúrbios no ciclo mitótico (LEME; MARIM-MORALES, 2009). Outra espécie utilizada é *Tradescantia pallida*, planta superior conhecida por ser altamente sensível aos agentes genotóxicos e adequada para o monitoramento ambiental da poluição atmosférica e contaminantes químicos em solo e água (CARNEIRO; TAKAYANAGUI, 2009).

Além das plantas, os peixes são muito utilizados como bioindicadores eficazes da contaminação aquática, uma vez que eles acumulam contaminantes e mostram uma resposta fisiológica, bioquímica, histológica e celular diferenciada (FONTANETTI et al., 2012). Tais organismos podem indicar variações na tolerância às condições ambientais produzidas pelo uso de agrotóxicos, incluindo alterações genéticas, constituindo assim excelentes bioindicadores, com uma ampla aplicação no monitoramento ambiental (YOHANNES et al., 2014).

Alterações no DNA, em eritrócitos de peixes, detetadas pelo ensaio do cometa e o teste do micronúcleo (MN) associado às anormalidades nucleares (AN), são biomarcadores muito empregados nos estudos de genotoxicidade (BOLOGNESI; HAYASHI, 2011; COLLINS, 2014). Também é conhecido que os efeitos de contaminantes em peixes podem se manifestar em órgãos e tecidos. O fígado é muito sensível a contaminantes ambientais tais como os agrotóxicos, sendo as células hepáticas consideradas como os primeiros alvos de toxicidade de uma substância (DE ALMEIDA; RIBEIRO, 2014). Assim sendo, ensaios histopatológicos e moleculares neste órgão constituem ferramentas importantes para estudar os efeitos tóxicos de compostos químicos como agrotóxicos. Análises de alterações histológicas e histoquímicas podem ser utilizadas para detectar os efeitos tóxicos diretos de agrotóxicos e são eficazes marcadores de estresses ambientais (SCHWAIGER et al., 1997; MAHROUS et al., 2015).

Biomarcadores bioquímicos e moleculares complementam os estudos de genotoxicidade e os histopatológicos, oferecendo maior informação sobre possíveis mecanismos da ação de agrotóxicos em organismos não alvos. A primeira resposta de um organismo frente a qualquer alteração induzida pelo estresse ambiental acontece a nível bioquímico. Estas respostas consistem em ativar sistemas de defesa antioxidante (tanto enzimático como não enzimático) e em proteínas de choque térmico (heat shock proteins-HSP) as quais são uma resposta primária de proteção (MILLER-MOREY; DOLAH, 2004).

Neste contexto, considerando o risco dos organismos não alvos frente ao emprego de agrotóxicos, estudos que avaliem a toxicidade e genotoxicidade do IMI, oferecendo mais informações sobre suas consequências ecotoxicológicas, são de extrema importância.

2. OBJETIVOS

O objetivo geral desse trabalho foi avaliar a toxicidade e genotoxicidade do inseticida imidacloprido em organismos não alvos, expostos a diferentes concentrações, tomando como base a concentração aplicada diretamente no solo da cultura de cana-de-açúcar, por meio de ensaios celulares e moleculares.

2.1 Objetivos específicos

- Avaliar a toxicidade, citotoxicidade e genotoxicidade do imidacloprido em células meristemáticas de *A.cepa* mediante a determinação dos índices de germinação, mitótico e de aberrações cromossômicas.
- Avaliar a genotoxicidade do imidacloprido em células da região F_1 de *A.cepa* mediante o teste do micronúcleo.
- Analisar o potencial genotóxico do imidacloprido em células reprodutivas de *T. pallida* por meio de teste do micronúcleo.
- Analisar o potencial genotóxico do imidacloprido em eritrócitos de *O. niloticus* por meio de ensaio do cometa e teste do micronúcleo associado à outras anormalidades nucleares.
- Avaliar a morfologia dos fígados de *O. niloticus* expostos ao imidacloprido, por meio de análise histológica.
- Detectar possíveis alterações na presença ou ausência de proteínas, lipídios e polissacarídeos no fígado de *O. niloticus* expostos ao imidacloprido, por meio de testes histoquímicos.
- Avaliar os efeitos das concentrações de imidacloprido na marcação de HSP70 em fígado de *O. niloticus* mediante imunohistoquímica.

3. REVISÃO DA LITERATURA

3.1 A problemática da contaminação ambiental por agrotóxicos no Brasil

Os agrotóxicos no Brasil foram primeiramente utilizados em programas de saúde pública (no combate de vetores e controle de parasitas), mas sua utilização intensiva na agricultura não aconteceu até a década dos anos 60 (OPAS, 1997). Desde esses anos até agora, o aumento das vendas de agrotóxicos no Brasil continua em ascensão, tornando-se hoje o maior mercado do mundo. O incremento das áreas para as culturas de cana-de-açúcar (estimados para 6 milhões de hectares em 2021/2022 no estado de São Paulo) (EGESKOG et al., 2014) está provocando um maior uso de agrotóxicos (VELASCO et al., 2012). Tem-se registrado no Brasil, 225 formulações empregadas neste cultivo, quase todas classificadas como altamente perigosas, especialmente para os sistemas aquáticos (FILOSO et al., 2015).

O uso indiscriminado de agrotóxicos tem efeitos negativos tanto na saúde das pessoas como no ambiente. A poluição pelos agrotóxicos em áreas agrícolas tem comprometido seriamente a qualidade dos recursos hídricos (águas superficiais e subterrâneas) afetando os ecossistemas aquáticos via escoamento superficial e lixiviação (ULRICH et al., 2013; BORTOLOZO et al., 2016). O risco ecológico de invertebrados e vertebrados aquáticos é alto, já que estes são grupos de organismos que cumprem funções importantes nos ecossistemas (BUNZEL et al., 2015; WANG et al., 2015).

Outro grupo de animais muito susceptíveis aos agrotóxicos, cuja população se encontra em declive, são as abelhas. Elas são altamente afetadas pelo uso de inseticidas, como os neonicotinóides (STONER; EITZER, 2012; SANDROCK et al., 2014; TAVARES et al, 2015) e já se encontra na literatura artigos alertando para a "crise da polinização" (GOULSON et al., 2015). A abundância, diversidade e saúde das comunidades da fauna selvagem do solo que desempenha um papel central na sua composição e funções ecológicas, também têm sido afetada negativamente pelo uso intensivo dos agrotóxicos (FRANCO et al., 2016). Outro grupo de organismos não alvos de ação de agrotóxicos, muito afetado pelo consumo direto e indireto destes químicos (SALA et al., 2010), são as aves, as quais oferecem importantes informações sobre a qualidade do ambiente e prevalência de poluentes que podem ser avaliados (ABBASI et al., 2016).

Os seres humanos estão indiretamente expostos a agrotóxicos por diferentes vias como sua produção, aplicação, ou ingestão de alimentos contaminados, sofrendo também o risco de padecer de seus efeitos tóxicos. Eles podem causar irritação cutânea e ocular e, em longos

períodos, problemas mais sérios, como efeitos no sistema nervoso, sistema reprodutivo, câmbios epigenéticos e câncer, afetando a saúde humana (COLLOTTA et al., 2013).

3.2 Neonicotinóides. Imidacloprido. Presença em solo e água

Os inseticidas neonicotinóides são agonistas de receptores acetilcolina nicotínicos (nAChRs) que formam parte de uma família de receptores de abertura e fechamento de canais iônicos, responsáveis pela neurotransmissão pós sináptica. Eles se ligam fortemente com estes receptores do sistema nervoso central de insetos, tendo efeitos semelhantes àqueles da nicotina, causando estimulação nervosa em baixas concentrações e o bloqueio do receptor, paralisia e morte em altas concentrações (JESCHKE et al., 2011).

Por serem compostos polares não voláteis e hidroliticamente estáveis, são facilmente absorvidos pelas plantas através de suas raízes ou folhas, e rapidamente transportados ao longo dos tecidos. Isto proporciona muitas vantagens no controle de pragas, proporcionando proteção para todas as partes da planta (GOULSON, 2013). Os neonicotinóides apresentam ainda uma grande diversidade estrutural, uma vez que se encontram compostos cíclicos e não cíclicos. De acordo com a sua estrutura química, podem ser classificados em três grupos: os N-nitroguanidinas (imidacloprido, tiametoxam, clotianidina e dinotefuran), os nitrometilenos (nitenpiram) e N-cianoamidinas (acetamiprida e tiacloprid) (NAUEN et al., 2001).

O IMI (1[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine), produzido pela “Bayer Crop Science” nos anos 90, foi o primeiro produto comercial de seu tipo (KOLLMEYER et al. 1999). A “U.S Environmental Protection Agency” (USEPA) o classifica como um “General Use Pesticide” (GUP) e de toxicidade II e III. Ele tem registro em mais de 120 países, sendo o inseticida mais eficaz para o controle de pragas como insetos sugadores do solo, pulgões, mastigadores e térmitas, cigarrinhas, fulgoromorfos, besouros, gorgulho, micro-Lepidoptera, coleópteros e nos mercados de sanidade animal (SUN; LIU, 2016). É utilizado em aproximadamente 140 culturas diferentes (JESCHKE; NAUEN, 2008), no Brasil em culturas de café, citros, batata, eucalipto, fumo, melão, pinus, cebola, feijão, tomate e cana-de-açúcar (ALAMOSBRASIL, 2015).

O ingrediente ativo IMI é registrado e comercializado no mundo em várias formulações com os nomes de: Confidor 70 WG, Gaucho 60 FS, Gaucho 70 WS, Jade 0.8 GR, Plural 20 OD, Couraze, Imidasect 5GR, Intercept, Merit Turf, Bamako 700 WG, Saluzi 600 FS, etc. (PESTICIDES REGISTRATION AND CONTROL DIVISION, 2015), sendo seu uso mais

comum o tratamento de sementes e aplicação em solo e folhagem (NATIONAL PESTICIDE INFORMATION, 2015).

Aproximadamente 90% dos inseticidas neonicotinóides entram no solo, e as quantidades variam dependendo da via de aplicação, tipo de solo e outros fatores ambientais. A vida média do IMI no solo é muito variável (28 e 1250 dias) e sua perda em áreas agrícolas é fundamentalmente através da degradação ou lixiviação nas águas (GOULSON, 2013), que apresenta fase inicial rápida e uma segunda fase, muito mais lenta (GUPTA et al., 2008).

Imediatamente após a aplicação, antes de ligar-se no solo, pode ocorrer à lixiviação dos neonicotinóides, de modo que níveis significativos podem ser previstos nas águas subterrâneas, particularmente se houver chuvas fortes nesse momento (THUYET et al., 2012). Em solo, o IMI é biotransformado, dependendo do ambiente, em vários metabólitos (6-chloronicotinic acid (CNA), 6-hydroxynicotinic acid, 6-methylmercaptonicotinic acid, chloronicotinic aldehyde, olefine-IMI, 5-OH-IMI, and 4-OH-IMI) até degradar-se totalmente em dióxido de carbono (BACEY, 2000), mas não ocorre da mesma forma na água. Eles não estão desenhados para seu uso nos corpos de água, assim a maior parte dele chega até a água por práticas de aspersão, escoamento ou lixiviação (MIRANDA et al., 2011), não sendo facilmente biodegradável (TISLER et al., 2009).

O IMI é estável em pH ácido e neutro e mais suscetível a hidrólise em águas muito alcalinas, sendo seu principal metabólito o 1-[(6-chloro-3-pyridinyl)methyl] -2-imidazolidone (ZHENG; LIU, 1999). A vida média de IMI em água (pH 7 e 25^{0}C) varia entre 33 e 44 dias (SARKAR et al., 1999). Portanto, a presença de IMI no meio aquático é cada vez mais preocupante, frente ao uso excessivo de produtos comerciais formulados com esta substância.

Vários estudos têm caracterizado a presença destes compostos nas águas. Por exemplo, em água de rios, riachos e drenos na Califórnia, foi detectado IMI em 89% das amostras, sendo que 19% das amostragens realizadas excedem o índice de referência estabelecido pela USEPA "Aquatic Life Benchmark" (STARNER; GOH, 2012). Também em rios de Sidney, Austrália, foram reportadas concentrações de IMI (SÁNCHEZ-BAYO; HYNE, 2014), maiores durante o tempo seco que durante os eventos de chuva (ENSMINGER et al., 2013).

As concentrações de IMI detectadas no ambiente aquático variam dependendo dos estudos e do método empregado e poucos são os estudos realizados no Brasil que avaliem os impactos em solo e água de agrotóxicos empregados no cultivo da cana-de-açúcar (SILVA et al., 2008; JACOMINI et al., 2011).

3.3 Estudos de toxicologia ambiental de neonicotinóides em organismos não alvos

Os agrotóxicos, além de serem concebidos para combater animais ou plantas consideradas pragas, podem chegar aos organismos não alvos, causando danos que devem ser avaliados. Estes, em especial os neonicotinóides, objeto de pesquisa deste trabalho, podem alcançar diferentes ambientes como solo, ar e água, fazendo-se necessários estudos em uma grande variedade de espécies de plantas e animais.

Os estudos de toxicologia ambiental tem se destacado muito nos últimos anos, devido a sua íntima relação com a proteção e recuperação dos ambientes. Para estes estudos, organismos bioindicadores como plantas, minhocas, abelhas, anfíbios, peixes, aves e mamíferos são muito utilizados.

A suscetibilidade dentro destes diferentes grupos de organismos varia, sendo os insetos mais sensíveis tanto por contato como por ingestão (CARVAJAL et al., 2012). Estes organismos são tipicamente mais suscetíveis que os vertebrados pelas diferenças nos receptores nAChR (MORRISSEY et al., 2015). Logo, muitos são os estudos realizados em diferentes espécies de insetos não alvos destes inseticidas neonicotinóides. Entre as espécies se encontram alguns insetos aquáticos, que tem evidenciado o perigo dos neonicotinóides em corpos de águas. Estudos dos efeitos do IMI foram realizados em larvas de insetos aquáticos da ordem díptera como *Chironomus riparius* (AZEVEDO-PEREIRA et al., 2011), *Caenis horaria* e *Chaoborus obscuripes* (ROESSINK et al., 2013), demonstrando as reduções de suas populações (DIJK et al., 2013).

Contudo, não só em insetos aquáticos os neonicotenóides têm drásticas consequências. Também se têm evidencia que a aplicação do IMI pode trazer um forte impacto sobre populações de abelhas em nível da colônia (GILL et al., 2012). No Brasil, o uso deste inseticida por meio de aplicação aérea tem sido associado à morte de abelhas em diferentes regiões do país, o que motivou a proibição de tal prática (IBAMA, 2015). Atualmente, permanece válido o processo de reavaliação e continua proibida sua aplicação por aviões, na época de floração e visitação de abelhas em qualquer tipo de cultura, frente aos efeitos nocivos observados (ALAMOSBRASIL, 2015). Vários são os estudos realizados em abelhas em que tem sido demonstrado que a exposição do IMI afeta a memória destes insetos, fazendo com que as abelhas operárias, após as atividades de forrageamento, não consigam retornar às suas colônias de origem o que pode levar ao desaparecimento desses animais (ROSSI et al., 2013; BRANDT et al., 2016).

Os vertebrados são menos sensíveis que os insetos, mas não estão livres de sofrer as consequências destes compostos (SÁNCHEZ-BAYO, 2012). Peixes da espécie *Paralichthys olivaceus* (SU et al., 2007) e *Danio rerio* (CROSBY et al., 2015) têm sido empregados para

avaliar a toxicidade do IMI. Estudos com outros organismos, como algumas famílias de aves insetívoras, mostraram reduções de suas populações pela presença do IMI (HALLMANN et al., 2014). As aves mostram toxicidade mais baixa, porém não deixam de estar expostas, uma vez que podem receber doses letais no consumo de sementes contaminadas com agrotóxicos durante as semeaduras (LOPEZ-ANTIA et al., 2013).

São múltiplos os estudos que avaliam a toxicidade de IMI em vários grupos de organismos, no entanto, poucos são os estudos que avaliam os danos no nível genético. O IMI pode apresentar potencial de indução de alterações genéticas em plantas e animais, sendo *A. cepa* e *T. pallida,* bons bioindicadores vegetais para detectar genotoxicidade (RAINO et al., 2010). Frequentemente, os testes com *A.cepa* têm sido muito utilizados para avaliação do potencial genotóxico de agrotóxicos como o IMI, devido à sua sensibilidade e boa correlação com outros sistemas testes (LIMAN et al., 2015; BIANCHI et al., 2016). Os ensaios com *T. pallida*, são menos utilizados, mas são altamente eficientes mostrando os efeitos dos agentes genotóxicos em células reprodutoras (PEREIRA et al., 2013).

Além de produzir alterações genéticas em plantas, o IMI também é genotóxico aos animais. Por exemplo, em anfíbios como *Hypsiboas pulchellus* (Anura, Hylidae) (PÉREZ-IGLESIAS et al., 2014), em minhocas *Eisenia fetida* (WANG et al., 2016) e peixes como *Channa punctatus* (PRIYA et al., 2014).

3.4 Bioindicadores vegetais. *Allium cepa* e *Tradescantia pallida* como organismos-teste em toxicologia ambiental

Plantas superiores são organismos utilizados mundialmente para o biomonitoramento ambiental, sendo excelentes modelos genéticos nestes estudos (HERRERO et al., 2012; LIMAN et al., 2015). Entre as espécies de plantas, *A. cepa* (Figura 1) tem sido utilizada desde os anos 40 com o objetivo de avaliar agentes químicos. Os primeiros usos de *A. cepa* como sistema teste, foram reportados no ano 1938 em pesquisas com colchicina e distúrbios no fuso mitótico (LEVAN, 1938). Esta planta apresenta características que a tornam um bom organismo teste como, por exemplo, cromossomos em número reduzido (2n=16) e de grande tamanho, ciclo celular bem conhecido e com grande número de células em divisão, fácil disponibilidade e rápido crescimento de raízes (EGITO et al., 2007; LEME; MARIN-MORALES, 2009). Além disso, suas vantagens na realização dos testes como baixo custo, fácil realização, não requerimento de sistemas metabólicos exógenos e possibilidade de empregar diferentes células e órgãos, fazem dela um excelente bioindicador (LEME; MARIN-MORALES, 2009).

O ensaio de *A.cepa* é usado para avaliar danos no DNA, tais como aberrações cromossômicas e distúrbios no ciclo mitótico (Figura 2). A medição de vários parâmetros como, por exemplo, os índices de germinação, mitótico e de aberrações cromossômicas permitem determinar a toxicidade, citotoxicidade e genotoxicidade em células meristemáticas de múltiplos compostos químicos. Além da determinação da frequência de micronúcleos na região F_1, que aporta grande informação sobre o potencial mutagênico dos compostos avaliados (LEME; MARIN-MORALES, 2009; GOUJON et al., 2015).

Tradescantia pallida (Figura 3) é outra planta superior que apresenta fácil adaptação em qualquer ambiente e pode se desenvolver durante o ano todo, tanto ao ar livre, nas regiões subtropicais, quanto em estufas, em qualquer parte do mundo. O tamanho, relativamente pequeno, e o código genético composto por seis pares de cromossomos (2n=12) relativamente grandes, tornaram essa planta um bioindicador favorável para estudos de genotoxicidade (ICHIKAWA, 1992). É conhecida por ser altamente sensível a contaminantes ambientais e adequada para o monitoramento de poluição atmosférica e contaminação química na água e solo. O teste do micronúcleo usando vários clones de *Tradescantia* (Trad-MCN), bem como a espécie *T. pallida,* tem sido amplamente utilizados para o monitoramento da genotoxicidade (MA et al., 1994; GUIMARAES et al., 2004). Quando é exposta a agentes genotóxicos, suas células reprodutoras apresentam fragmentos do núcleo (micronúcleos) que se formam a partir de quebras cromossômicas ou perda de cromossomos, refletindo danos no nível cromossômico (Figura 4) (CARNEIRO; TAKAYANAGUI, 2009), sendo então utilizada como ferramenta no monitoramento ambiental (MIELLI et al., 2009; GALVÃO et al., 2014).

Figura 1: *Allium cepa* (Amarilidaceae). Foto google.

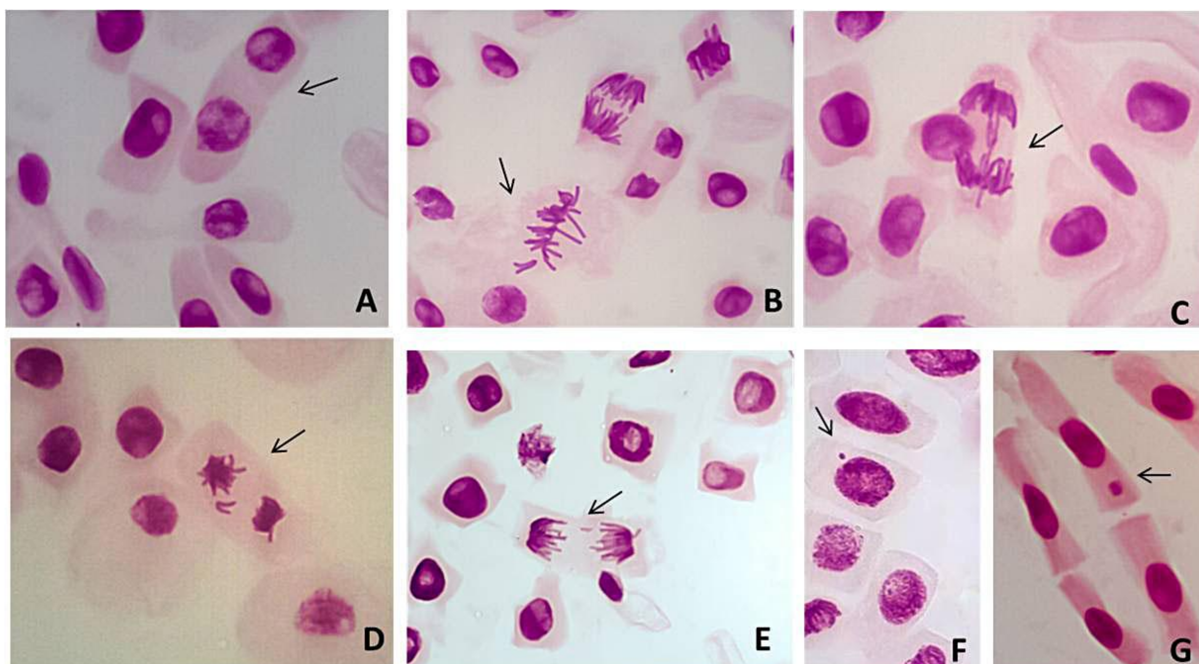

Figura 2: Danos no DNA de células de *A. cepa* expostas ao IMI; (A) células binucleadas (seta); (B) metáfase com perda cromossômica (seta); (C) anáfase com ponte cromossômica (seta); (D) anáfase com perda cromossômica (seta); (E) anáfase com ruptura cromossômica (seta); (F) celula meristemática na intérfase com micronúcleo (seta); (G) intérfase com micronúcleos na região de F_1 (seta). Ampliação 400X. Fotos da autora.

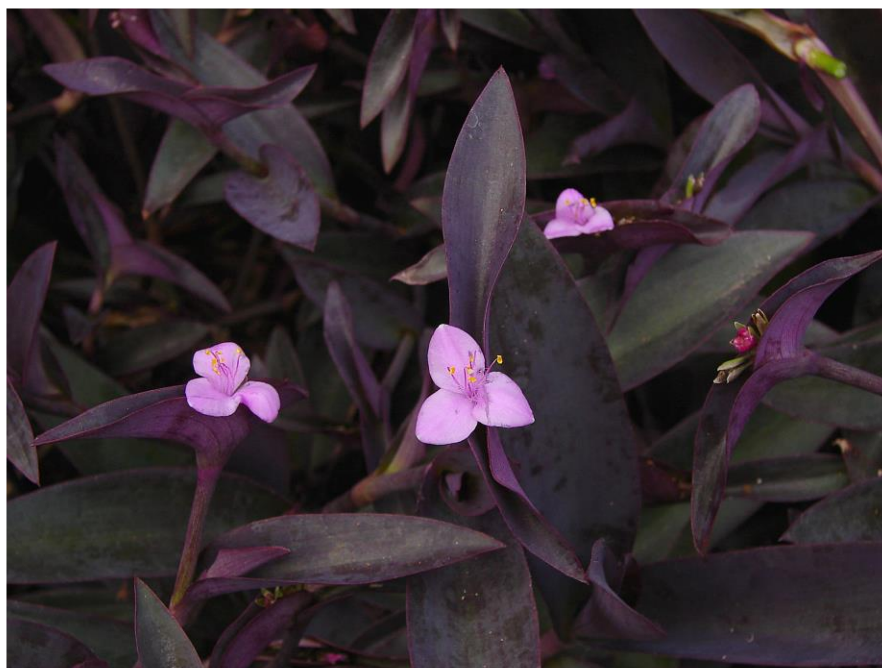

Figura 3: *Tradescantia pallida.* Foto da autora.

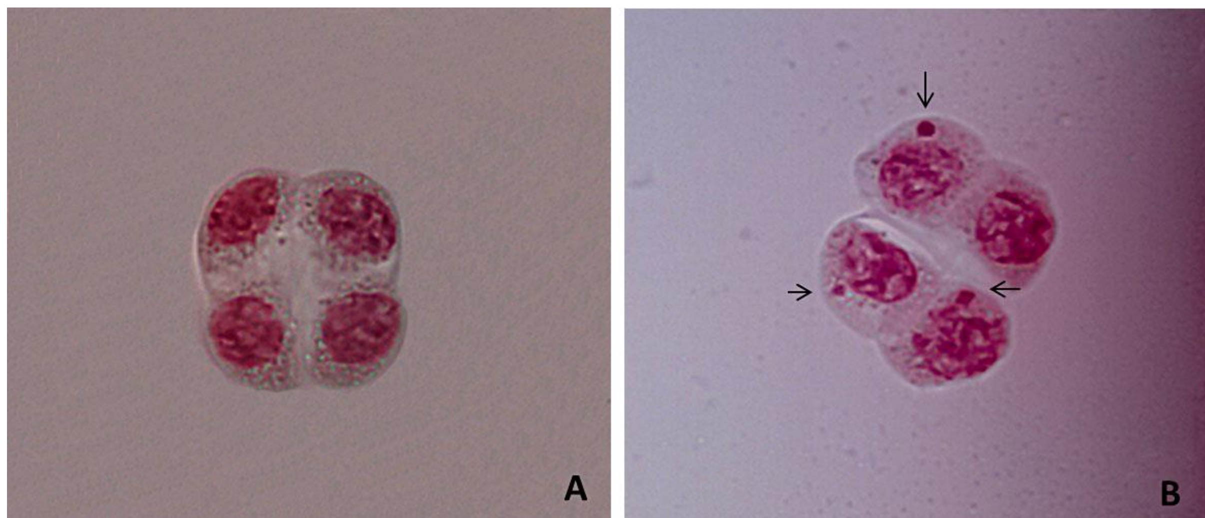

Figura 4: Fotografía de uma tétrade normal de uma célula de *T. pallida* (A) e uma tétrade expostas ao IMI com vários micronúcleos (setas) (B). Ampliação 400X. Fotos da autora.

3.5 *Oreochromis niloticus* (Perciformes: Cichlidae). Bioindicador para o monitoramento ambiental

Os peixes são muito empregados no monitoramento ambiental, sendo modelos eficazes da contaminação aquática. Entre as espécies mais utilizadas encontra-se *O. niloticus*, conhecida como tilapia do Nilo, a segunda espécie de peixe mais cultivada no mundo (NG; ROMANO, 2013) e mais importante na aquicultura do século XXI (FITZSIMMONS, 2013). Tem sido considerada ótima em estudos laboratoriais por suas múltiplas vantagens como alta taxa de crescimento e reprodução, resistência e tolerância nas condições de laboratório e adaptação na alimentação comercial, além de ser um modelo confiável por ser extremamente vulnerável a agressão tóxica de produtos químicos (GARCIA-SANTOS et al., 2006; KENAWY et al., 2009). São de fácil aquisição em sistemas de aquicultura no Brasil, sendo muito utilizado neste país em pesquisas de ecotoxicologia (FUZZINATO, 2013).

Os estudos com peixes fornecem a possibilidade de avaliar danos em vários órgãos e tecidos e ter respostas celulares, bioquímicas e moleculares mais completas sobre os efeitos dos agrotóxicos. Entre os tecidos e órgãos mais utilizados estão o sangue e o fígado. Os eritrócitos são células facilmente coletados sem sacrificar os animais e não requerem métodos de separação adicional (CAVAS, 2011). O fígado é um órgão essencial, devido ao seu alto metabolismo, desintoxicação de produtos endógenos e exógenos tais como agrotóxicos, e muito sensível aos contaminantes ambientais. As células hepáticas são consideradas alvos principais da toxicidade de uma substância (DE ALMEIDA; RIBEIRO, 2014). Logo,

biomarcadores celulares e moleculares aplicados em vários órgãos de peixes proporcionam uma melhor compreensão sobre os diferentes danos e mecanismo de indução de agrotóxicos (LAWRENCE; HEMINGWAY, 2007).

3.5.1 Estudos de genotoxicidade. Ensaio do cometa e teste do micronúcleo.

São múltiplos os biomarcadores que podem ser avaliados utilizando peixes como bioindicadores. Entre eles, estudos de genotoxicidade usando biomarcadores que detectem danos no nível primário e cromossômico da molécula de DNA como, por exemplo, ensaio do cometa e teste do micronúcleo, são recomendados.

O ensaio de eletroforese alcalina em gel com células individuais, mais conhecido como ensaio do cometa, detecta rupturas de cadeia simples e dupla, sítios álcali-lábeis e com atraso na reparação como danos iniciais (COLLINS, 2014). Este ensaio tem sido muito utilizado em estudos de genética ecotoxicológia. Uma revisão sobre este tema foi publicada pelos autores e encontra-se no Capítulo 5 (ANSOAR-RODRÍGUEZ et al., 2015).

Outro ensaio aplicado por mais de 30 anos nestes estudos é o teste do micronúcleo (MN) associado às anormalidades nucleares (AN), sendo uns dos mais promissores ensaios em ecotoxicologia para a detecção de dano ao nível cromossômico (CARRASCO et al., 1990; BOLOGNESI; HAYASHI, 2011). Os MN são pequenos corpos extranucleares que aparecem durante o processo de divisão celular. Podem-se desenvolver de forma espontânea e/ou induzido por agentes clastogênicos e aneugênicos (MELO et al., 2013). Os MN formados por agentes clastogênicos provem de fragmentos de cromossomos ou cromátides, enquanto os de origem aneugênica são formados por cromossomos inteiros. Estes fragmentos ou cromossomos inteiros, não são incluídos no núcleo das células filhas na telófase, sendo rodeados por envoltório nuclear assumindo a morfologia de um núcleo interfásico bem menor que o núcleo principal (AL-SABTI; METCALFE, 1995; FENECH et al., 2011; KIRSCH-VOLDERS et al., 2011).

Os MN podem ter diferentes destinos, entre eles, a reintegração da cromatina do MN no núcleo principal da célula (EGE et al., 1974), a retenção do MN na célula até a divisão celular seguinte (HOFFELDER et al., 2004) a expulsão do MN da célula, o que levaria à perda do material genético contido no MN (RAO et al., 2008) e alguns MNs cujo DNA não pode ser reparado, terminam se degradando (TERRADAS et al., 2010).

Além dos MN, algumas alterações morfológicas no envoltório nuclear dos eritrócitos de peixes foram descritas e podem complementar estes estudos (CARRASCO et al., 1990). Estas

alterações nucleares em eritrócitos podem ser atribuídas ao dano em membranas causado por espécies reativas de oxigênio (EROs) (AHMAD et al., 2006). A frequência nestas alterações varia dependendo de vários fatores como natureza do agente tóxico, sua cinética, espécie de peixe, tempo de exposição e ciclo hematopoiético, além de estarem relacionadas com a formação de MN (CHEONG et al., 2011).

O teste do MN associado à outras alterações nucleares tem sido muito aplicados em uma grande variedade de peixes, para estudos de genotoxicidade e de monitoramento ambiental de diferentes químicos (HARABAWY, MOSLEH, 2014; OBIAKOR et al., 2014; TORRE et al., 2015) sendo bons biomarcadores de efeitos genotóxicos. Estes ensaios são reconhecidos devido à sua robustez, sensibilidade e repetividade para avaliar danos ao DNA, sendo uma excelente combinação para estes estudos (KANG et al., 2013).

3.5.2 Histopatologia do fígado como biomarcador para avaliação da poluição aquática

O fígado dos peixes apresenta hepatócitos dispostos como túbulos em forma piramidal com suas bases dirigidas para os sinusóides (HAMPTON et al., 1988). As células hepáticas são grandes, com forma poliédrica; apresenta núcleos redondos bem definidos com um nucléolo característico, intercalados por sinusóides, nos quais se observam eritrócitos (TORRES et al., 2010). O citoplasma das células hepáticas contém grande quantidade de mitocôndrias, retículo endoplasmático rugoso e grande quantidade de glicogênio (SAMANTA et al., 2015). O fígado é um órgão metabolizador de todas as substâncias que vem pela via sanguínea. Em peixes desempenha um papel chave na desintoxicação xenobiótica através da oxidação, redução, conjugação e reações de hidrólise que fazem o armazenamento ou a excreção de toxinas (MAKSYMIV et al., 2015). Os efeitos tóxicos de produtos químicos, normalmente podem ser observados no fígado, fornecendo dados importantes sobre a toxicidade do produto químico e modo de ação (HINTON; LAUREN, 1990; DE ALMEIDA; RIBEIRO, 2014). É considerado um órgão alvo de grande importância para as análises do dano tissular causado por substâncias como os agrotóxicos (AMARAL et al., 2002).

As alterações histológicas são ferramentas úteis para avaliações das relações entre danos em peixes e poluição ambiental, atuando como bons marcadores de estresses ambientais (DYK et al., 2012; MAHROUS et al., 2015). Para as análises destas alterações histológicas em diferentes órgãos de peixes pode ser utilizado um protocolo semi-quantitativo; a aplicação deste protocolo oferece informações importantes sobre a funcionalidade do tecido, tendo em

conta os diferentes tipos de alterações, frequência e o fator de importância delas (BERNET et al., 1999; MARINHO et al., 2014).

Inúmeros são os estudos histológicos em peixes expostos a diferentes agrotóxicos, como por exemplo, a exposição aguda ao inseticida fenitrothion em *O. niloticus* (BENLI; OZKUL, 2010), o tratamento crônico de diazinon em *Oncorhynchus mykiss* (BANAEE et al., 2013), a exposição crônica ao herbicida metsulfuron metil e chlorimuronetil em *Anabas testudineus* (SAMANTA et al., 2015) e a exposição a curto prazo de pendimetalina em *Channa punctata* (TABASSUM et al., 2016).

3.5.3 Ensaios bioquímicos e moleculares. Ferramentas sensíveis na determinação de estresse ambiental

Os organismos apresentam mecanismos que lhes conferem proteção frente ao estresse ambiental, induzido por diferentes fatores, como a exposição aos agrotóxicos. Estes mecanismos consistem em ativar sistemas de defesa antioxidante e proteínas de choque térmico (MILLER-MOREY; DOLAH, 2004).

As proteínas de choque térmico (HSP) são um grupo de chaperonas moleculares filogeneticamente altamente conservadas que desempenham um papel integral das vias de resposta ao estresse celular na maioria dos organismos, incluindo peixes. Sendo assim, são amplamente utilizados como biomarcadores de exposição a estressores ambientais (DEANE; WOO, 2011; CHADWICK et al., 2015).

As HSPs podem ser classificadas em cinco grandes famílias baseando-se em seu peso molecular, homologias na sequência de aminoácidos e funções. Entre elas encontramos as HSP100, 90, 70, 60 KDa e a família de menor tamanho denominadas como HSPs (JOLY et al., 2010). As HSP70 e HSP90 são as proteínas mais extensivamente estudadas e conservadas destas famílias. Elas desempenham papéis importantes na célula como chaperonas moleculares, degradação de proteínas mal dobradas e outros processos regulatórios (LI; DU, 2013). As proteínas HSP70 e HSP90 podem ser induzidas por vários fatores de estresse ambiental, como exposição aos agrotóxicos e funcionar como ativadores potentes da desintoxicação metabólica (COLINET et al., 2010; XU; QIN, 2012).

Os membros da família de 70 kDa (HSPs70) são um grupo de moléculas evolutivamente muito conservadas numa variedade de organismos vivos e distribuídas ubiquamente (RICHTER et al., 2010). São as mais estudadas, com 40.000 artigos publicados até este ano (METZGER et al., 2016). Elas protegem as células dos danos causados por uma grande

variedade de estímulos estressantes (LINDQUIST; CRAIG, 1988; LIU et al., 2015). HSP70 é uma chaperona molecular que está implicada numa ampla variedade de processos celulares importantes como: biogênese de proteínas, proteção do proteoma frente ao estresse, recuperação de proteínas agregadas e facilitação da translocação de proteína através das membranas, e demais funções especializadas, como desmontagem de complexos protéicos (CLERICO et al., 2015). É outro sistema de proteção dos organismos frente ao estresse oxidativo, impedindo a perda irreversível de proteínas vitais e facilitando sua subsequente regeneração (HE et al., 2010).

A primeira resposta ao estresse celular é uma resposta geral caracterizada por um aumento das proteínas de estresse, assim, a expressão de HSP é comumente utilizada como indicador de estresse celular em animais. O sinal intracelular para a indução de genes hsp é um aumento súbito de estresse induzido por polipeptídeos anormais no citosol ou núcleo (SHERMAN, GOLDBERG, 2001). A maior concentração de HSP70 hepático é citosólico, mas, ele pode ser induzido pela exposição de tóxicos e se localizar no núcleo e na membrana mitocondrial (WEBER et al., 2002).

No nível ecológico, HSP70 protege os organismos de vários estresses ambientais como calor, frio, dessecação, toxinas, patógenos e outros (KREGEL, 2002). Portanto, os níveis de transcrição e tradução de proteínas codificadas por genes hsp70 podem ser utilizados como biomarcadores para monitorar as respostas celulares e fisiológicas frente a diversos estímulos ambientais (NAZIR et al., 2003). Em peixes, os genes hsp70 podem ser induzidos pela exposição à agrotóxicos (Figura 5). A expressão destas proteínas podem estar relacionada com o estresse oxidativo ou por alterações na indução de citocinas pró-inflamatórias decorrente da exposição à agrotóxicos (EDER et al., 2004; XING et al., 2013).

O estresse oxidativo pode induzir expressão de mRNA de HSP70 na fase inicial, indicando que os radicais de oxigênio, especialmente ânions superóxido, contribuem para a expressão de HSP70 (KUKREJA et al., 1994). HSP70 protege as células da ação de espécies reativas de oxigênio (EROS) por meio de uma variedade de proteínas envolvidas em mecanismos moleculares anti-estresse. As EROS causam a acumulação de proteínas oxidadas, carboniladas ou ubiquitinadas que são substratos típicos das chaperonas HSP70 (CASTRO; OTT, 2012). Aliás, as HSP70 podem modular a atividade de proteínas que participam na sinalização induzida por EROS e apoptose (LUO et al., 2011). Alguns autores referem à indução da expressão dessa proteína, baixas condições de estresse causadas por agrotóxicos como, por exemplo, atrazina e clorpirifo (XING et al., 2013), sendo a determinação de HSP70 uma ferramenta importante para este estudo.

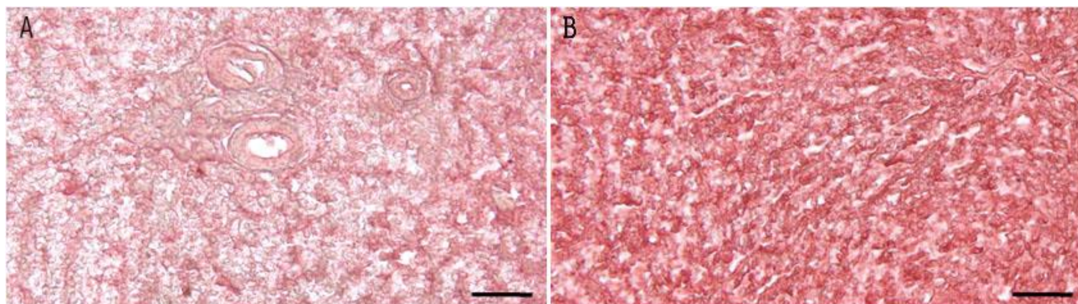

Figura 5: Imunomarcação de HSP70 no fígado de *O. niloticus*. Grupo controle (A) e Grupo exposto ao IMI (B): barra: 20 µm. Foto da autora.

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5. RESULTADOS

Durante a realização desta tese foram produzidos quatro artigos, dos quais três já se encontram publicados e o último está aceito.

5.1 ARTIGO 1. Aplicaciones del Ensayo Cometa en genética ecotoxicológica (ANEXO 1)

ANSOAR-RODRÍGUEZ, Y.; FONTANETTI, C.S.; CHRISTOFOLETTI, C.A.; DÍAZ-LLERA, S.C.

Publicado na **Revista CENIC Ciencias Biológicas**, v. 46, n. 1, p. 51-62, 2015.

5.2 ARTIGO 2. *Allium cepa* and *Tradescantia pallida* bioassays to evaluate effects of the insecticide imidacloprid (ANEXO 2)

ANSOAR-RODRÍGUEZ, Y.A.; CHRISTOFOLETTI, C.A.; PEDRO-ESCHER, J.; BUENO, O.C.; MALASPINA, O.; FERREIRA, R.F.C.; FONTANETTI, C.S.

Publicado na **Chemosphere**, v. 120, p. 438-442, 2015.

5.3 ARTIGO 3. Genotoxic potential of the insecticide Imidacloprid in a non-target organism (*Oreochromis niloticus*-Pisces) (ANEXO 3)

ANSOAR-RODRÍGUEZ, Y.; CHRISTOFOLETTI, C.A.; MARCATO, A.C.; CORREIA, J.E.; BUENO, O.C.; MALASPINA, O.; FONTANETTI, C.S.

Publicado no **Journal of Environmental Protection**, v. 6, p. 1360-1367, 2015.

5.4 ARTIGO 4. Liver alterations in *Oreochromis niloticus* (Pisces) induced by insecticide imidacloprid: histopathology and heat shock protein *in situ* localization (ANEXO 4)

ANSOAR-RODRÍGUEZ, Y.; CHRISTOFOLETTI, C.A.; CORREIA, J.E.; DE SOUZA, R.B.; DE SOUZA, C.M.; MARCATO, A.C.; BUENO, O.C.; MALASPINA, O.; SILVA-ZACARIN, E.C.M.; FONTANETTI, C.S.

Artigo aceito no **Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes** (Ms # B-2192).

6. CONSIDERAÇÕES FINAIS

Atualmente há uma grande demanda em nível mundial dos combustíveis "verde" como uma alternativa dos combustíveis fóssil. O impacto ambiental na utilização de cultivos para obtenção de biocombustíveis está sendo discutido, principalmente, devido à perda de habitats, degradação dos solos e aumento descontrolado no emprego de agrotóxicos. Neste contexto, o Brasil lidera hoje o ranking mundial no cultivo de cana-de-açúcar para obtenção de bioetanol e também no consumo de agrotóxicos. Entre as 225 formulações destes químicos registradas para sua utilização na cultura de cana-de-açúcar, encontra-se o IMI, inseticida que põe em risco organismos não alvo de sua ação.

O presente trabalho avaliou os efeitos do IMI em organismos não alvos de açao deste inseticida. A partir da dose de IMI aplicado no solo na cultura de cana-de-açúcar (400 g/ha) foram determinadas as concentrações avaliadas neste estudo.

As avaliações realizadas em células meristemáticas e da região F_1 de *A.cepa*, demostraram a eficiência deste teste para avaliar o potencial tóxico, citotóxico e genotóxico do IMI. A toxicidade foi conferida por meio do índice de germinação das sementes de *A. cepa*, não sendo o IMI tóxico para estas células, com uma porcentagem de sementes germinadas igual ao controle negativo. Não foi observada citotoxicidade do composto determinada pelo índice mitótico das células meristemáticas, uma vez que não houve aumento e/ou diminuição deste índice para as concentrações testadas. Porém, o índice de aberrações cromossômicas teve um aumento dose-dependente visualizado em todas as fases do ciclo celular. Foram observadas alterações como: células binucleadas, metáfase com perda cromossômica, anáfase com ponte, perda e quebra cromossômica e intérfase com MN, mostrando o potencial genotóxico do IMI. É conhecido que a principal causa das alterações nas estruturas cromossômicas está relacionada com quebras do DNA e inibição da replicação e sua síntese. Isto pode acontecer de forma espontânea ou induzida por agentes químicos como agrotóxicos. Além destas alterações nas células meristemáticas de *A. cepa*, foi encontrado um aumento na freqüência de MN em células de região de F_1 no grupo exposto a mais alta concentração de IMI. Isto demonstra a permanência do dano observado nas células meristemáticas, nas células da região de F_1. As três concentrações de IMI, além de genotóxicas em *A. cepa*, também causaram um aumento na freqüência de MN das células reprodutivas de *T. pallida*. Estes resultados alertam para o risco do IMI causar dano genético em organismos não alvos como as plantas superiores.

Devido à presença do IMI em corpos de águas, também foi avaliado o risco em organismos aquáticos. A fácil extração dos eritócitos do sangue periférica permitiu-nos avaliar o dano primário e cromossômico nestas células. Mediante o ensaio do cometa alcalino, foi observado que as três concentrações de IMI induziram dano primário em eritrócitos de *O. niloticus,* sendo portanto, genotóxico para a espécie. Estes resultados são semelhantes aos observado nas plantas avaliadas. O teste do MN demostrou que só a maior concentração provocou danos estatisticamente significativos em nível cromossômico, observado pelo aumento da freqüência de MN e de anormalidades nucleares como "blebbed nuclei" e "notched nuclei". Os resultados sugerem que o dano primário induzido pelo IMI nas três concentrações avaliadas foi reparado, com exceção da concentração mais alta. O ensaio do cometa e o teste do MN constituem boa combinação para avaliar efeitos genotóxicos em diferentes níveis.

O dano genotóxico observado tanto em plantas superiores como em peixes poderia estar relacionado com estresse oxidativo. As EROS podem interagir com biomoléculas como o DNA e resultar em um desequilíbrio entre as moléculas pró-oxidantes e os mecanismos de defesa antioxidantes.

Além dos eritrócitos, foram avaliadas outras células em peixes, como os hepatócitos, alvos da toxicidade de uma substância. Foram registradas alterações nos hetapócitos dos peixes tratados nas três concentrações de IMI, sendo a degeneração hidrópica, os núcleos picnóticos e a perda do limite celular, as mais encontradas. Nos animais expostos à concentração mais alta, foi observado um aumento de lipídeos no citoplasma dos hepatócitos (esteatose). Na maioria das espécies de peixes, os lipídios são fontes predominantes para a obtenção de energia, mas sua deposição excessiva pode causar problemas para a saúde do animal. A exposição ao estresse ambiental pode perturbar o metabolismo de lípidos, observando-se um acúmulo no fígado.

Muitos destes danos estão relacionados com estresse oxidativo e, portanto, pode acontecer uma ativação dos sistemas de defesa antioxidantes e proteínas de choque térmico que são uma resposta protetora primária. Neste contexto, foi observada uma alta inmunoreação de HSP70 no fígado dos animais expostos a maior concentração de IMI.

Diante do exposto, ressaltamos que os estudos realizados neste trabalho contribuem com informações sobre o risco deste inseticida em organismos não alvos. Assim, devemos continuar avaliando o risco e as possíveis estratégias para alcançar um equilíbrio entre a utilização dos neonicotinóides para atender as demandas na produção de alimentos e

combustíveis e a necessidade de gestão da biodiversidade do mundo para garantir a saúde dos ecossistemas.

7. CONCLUSÕES

- As concentrações testadas de IMI não foram tóxicas nem citotóxicas às células meristemáticas de *A. cepa*.

- O aumento do índice de aberrações cromossômicas em células meristemáticas de *A. cepa* e na freqüência de MN em *T. pallida*, nas concentrações testadas de IMI, demonstraram o potencial genotóxico deste inseticida.

- A maior concentração de IMI mostrou um aumento na freqüência de MN em células da região F_1 de *A. cepa*, sugerindo a permanência do dano encontrado nas células meristemáticas.

- As concentrações testadas de IMI causaram dano em nível primário em eritrócitos de *O. niloticus* e a maior concentração, dano em nível cromossômico com um aumento na freqüência de MN.

- As alterações mais freqüentes encontradas em fígados de *O. niloticus* expostos ao IMI foram degeneração hidrópica, núcleo picnótico e perda do limite celular.

- A maior concentração de IMI mostra esteatose nos hepatócitos de *O. niloticus* e aumento na expressão da proteína de choque térmico HSP70.

- Concluímos que se deve ter muita cautela no uso do neonecotinóide IMI, uma vez que foi detectado seu potencial tóxico em vários níveis nos diversos organismos testados.

ANEXOS

ANEXO 1

Aplicaciones del Ensayo Cometa en Genética Ecotoxicológica

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Keywords: cometa assay, ecotoxicology, genotoxic agents, environmental biomonitoring.

RESUMEN. El ensayo de electroforesis alcalina de células individuales embebidas en microgel, más conocido como Ensayo Cometa (EC), ha logrado ubicarse en una posición privilegiada dentro de los ensayos utilizados para evaluar la respuesta de las células frente a agentes genotóxicos, estreses bióticos y abióticos que conducen a daño al ADN. Diferentes versiones del ensayo se han desarrollado con el fin de ampliar el espectro de las lesiones al ADN que pueden ser detectadas. Igualmente, se han proporcionado directrices para su uso en la genética ecotoxicológica. El EC se distingue por su demostrada sensibilidad para detectar bajos niveles de daño, su rapidez y simplicidad en el análisis de los datos a nivel de células individuales, el pequeño tamaño de muestra requerido, su flexibilidad, bajo costo y aplicabilidad a cualquier población de células. Este grupo de ventajas justifica su amplio uso en la evaluación genotóxica *in vitro*, de productos químicos industriales y agroquímicos, fármacos, principios activos, así como en el biomonitoreo ambiental y humano de sustancias genotóxicas. La gran diversidad de organismos en los cuales este ensayo puede ser aplicado, aumenta su espectro de utilidad y aplicación creciente en ecotoxicología. Por tanto, en esta revisión se aborda el empleo del EC en los principales modelos de organismos (plantas, protozoos, pequeños crustáceos, insectos, lombriz de tierra, moluscos, peces, anfibios y mamíferos silvestres) utilizados en estudios de ecotoxicología. La gran variedad de estudios que utilizan este ensayo y sus múltiples ventajas para detectar efectos genotóxicos en organismos de diferentes ecosistemas, lo hacen muy recomendable para análisis ecotoxicológicos.

ABSTRACT. The single cell gel electrophoresis assay, better known as Comet Assay (CA), has obtained a privileged position among the tests used to assess the response of cells to genotoxic agents, biotic and abiotic stresses which lead to DNA damage. Different versions of the assay have been developed aimed to widen the spectra of DNA lesions that can be detected. Guidelines have also been provided for its use in ecotoxicological genetics. CA stands out by its demonstrated sensitivity to detect low levels of DNA damage, speediness and straightforwardness

for data analysis in single cells, the use small size samples, its flexibility, low cost and applicability to almost any cell population. This set of advantages justifies its wide use for the genotoxicological evaluation *in vitro* of industrial chemicals, agrochemicals, pharmaceuticals, active principles, as well as for the environmental and human biomonitoring of genotoxicants. The broad variety of organisms where this assay could be applied increases its range of utility and applicability in ecotoxicology. Therefore, this review deals with the use of the CA in the main model organisms (plants, protozoa, small crustaceans, insects, earthworms, mollusks, fish, amphibians and wild mammals) used in ecotoxicology studies. The wide variety of studies using this assay and its many advantages to detect genotoxic effects in organisms of different ecosystems, make it highly suitable for ecotoxicological analysis.

INTRODUCCIÓN

Diversos millares de productos químicos tales como metales, agroquímicos, aditivos alimenticios, desechos industriales y colorantes entre otros, son regularmente liberados al ambiente por el hombre.[1] Todos los organismos de la biósfera, están diariamente expuestos a muchos de estos productos y otros agentes físicos, químicos o biológicos presentes en el medio. El desarrollo industrial acelerado con sus residuos y la combustión incompleta de hidrocarburos, más la amplia utilización de productos agroquímicos, constituyen un riesgo de inestabilidad genética para aquellos organismos que viven en los ambientes afectados. Algunos de estos agentes pueden generar rupturas de cadenas de ADN, modificaciones de bases nitrogenadas y enlaces cruzados que, como consecuencia del metabolismo celular, podrían derivar en mutaciones génicas o reordenamientos cromosómicos y, consecuentemente, en alteraciones del metabolismo, la sobrevivencia y la proliferación celular.[2] Estas mutaciones, a su vez, pueden traer severas consecuencias para las poblaciones, las especies y la estabilidad del ecosistema.[3]

La vigilancia de los contaminantes genotóxicos en el ambiente tiene dos propósitos: analizar su riesgo para los organismos que habitan normalmente en dicho medio y la evaluación del riesgo a su exposición para los humanos en diferentes modelos biológicos. Tal es el caso del cultivo de alimentos en suelos contaminados u obtenidos con agua contaminada.[4] Esta vigilancia se realiza mediante el empleo de una batería de ensayos para detectar diferentes niveles de daño genotóxico.[5] El ensayo de electroforesis alcalina de células individuales embebidas en microgel, más conocido como Ensayo Cometa (EC) es una metodología sensible, simple, rápida, económica y aplicable a cualquier tipo celular para medir rupturas de cadena de ADN y sitios lábiles a álcali a nivel de células individuales. Es considerado como un ensayo indicador para detectar lesiones premutagénicas y puede ser usado para estudios mecanísticos. Se ha sugerido su uso *in vivo* como ensayo de seguimiento apropiado para investigar la significación de los resultados de genotoxicidad positivos *in vitro*.[6,7] Por su utilidad, se han publicado guías internacionales para la conducción de este ensayo y se espera que sea incluido en las guía de la Organización para el Desarrollo y la Cooperación Económica (OECD por sus siglas en inglés) para 2014.[8] Otras revisiones sobre la aplicación de este ensayo en ecotoxicología han sido publicadas con diferentes enfoques.[9, 10] Esta revisión aborda el empleo del EC en diferentes especies de organismos modelo utilizados para estos estudios.

Ensayo cometa

En 1978, Rydberg y Johanson, describieron este método para el análisis de células individuales irradiadas, basado en la lisis alcalina.[11] Este fue más tarde modificado incorporando los microgeles de agarosa y la electroforesis.[12] Después de subsecuentes intentos para aumentar la sensibilidad y aplicabilidad del ensayo, se consolidaron dos versiones diferentes de él. La versión alcalina,[13] en la que se aumentó considerablemente el pH ($pH > 13$) para permitir la detección de las roturas de simple cadena y sitios lábiles a álcali, además de las de doble cadena como daños iníciales, y la versión neutra,[14] que solo detecta roturas de doble cadena. La versión alcalina fue

recomendada como la versión óptima del ensayo para la identificación de sustancias con actividad genotoxicológica potencial en el *International Workshop on Genotoxicity Test Procedures* (IWGTP).[15] Varios autores tienen descritos protocolos del EC con numerosas variantes, sistemas celulares, múltiples órganos y enzimas de reparación.[16-18] Este ensayo detecta ruptura de cadenas que pueden ser generadas en la célula por una variedad de agentes químicos (por ejemplo, especies reactivas de oxígeno), físicos (como las radiaciones ionizantes) y procesos celulares (como la respiración y la reparación del ADN).[17]
De todos los ensayos de estimación de daño primario al ADN, este es el más sensible para detectar bajos niveles de daño, por su rapidez, su simplicidad en el análisis de los datos a nivel de células individuales, por requerir un pequeño tamaño de muestra, su flexibilidad, su bajo costo y su aplicabilidad a cualquier población de células.[15,17] Estas ventajas justifican su amplio uso *in vitro* e *in vivo* en la evaluación genotóxica de sustancias químicas, así como en el biomonitoreo ambiental y humano.[17,19,20]
El biomonitoreo ambiental mediante el EC, permite hacer uso de organismos vivos o “centinela" en su hábitat natural y evidenciar la exposición continua a corto y largo plazo. En los últimos años, el EC se ha aplicado en un gran grupo de organismos filogenéticamente heterogéneos que incluyen: plantas inferiores y superiores, oligoquetos, poliquetos, planarias, crustáceos, insectos, bivalvos, gasterópodos, asteroides y equinoideos, peces, anfibios, reptiles y mamíferos.[4] Estos estudios han empleados una gran variedad de agentes químicos y tipos celulares, en condiciones de laboratorio y de campo.[19,21] El principio de esta técnica es el mismo, solo que el tratamiento de la muestra varía en dependencia del organismo a utilizar para el ensayo. Esta revisión se ha enfocado hacia la aplicación del ensayo en células de los organismos modelos más empleados en los estudios de ecotoxicología.

EL ENSAYO COMETA EN DIFERENTES MODELOS EXPERIMENTALES

Plantas

Las plantas son marcadamente sensibles a los contaminantes ambientales, incluidos los productos químicos y metales pesados. En particular, las hojas representan una diana ideal para los propósitos de biomonitoreo, pues en ellas se pueden acumular grandes cantidades de contaminantes. Actualmente, existe un interés marcado en la sustitución de los modelos animales por modelos vegetales en la investigación farmacológica y toxicológica. Por tanto, esta técnica se ha convertido en una herramienta útil para el uso de las plantas superiores como sensores estables en los ecosistemas y fuente de información sobre los efectos genotóxicos de contaminantes.[22] La combinación del EC con la detección *in situ* de especies reactivas del oxígeno (ERO) inducida por los rayos *gamma*, puede ser usada para obtener información sobre el nivel de radiosensibilidad de diferentes genotipos.[22] El EC se puede utilizar para evaluar el potencial de fitorremediación de especies o genotipos de plantas hacia compuestos genotóxicos específicos y suelos contaminados. Una aplicación del EC que ha tenido éxito en el campo de la fitorremediación es la evaluación de la actividad genotóxica de las cenizas volantes, un subproducto de las centrales termoeléctricas cuya eliminación requiere de grandes cantidades de tierra y agua.[23] Es una fuente de información útil para evaluar la contaminación en el suelo, el agua y el aire mediante el análisis de diferentes tejidos de las plantas (raíces, tallos, hojas).[24] Este ensayo ha sido aplicado para la evaluación del efecto de los metales pesados,[25,26] de contaminantes en el aire,[27] de las radiaciones ionizantes y no ionizantes,[28-30] y de contaminantes orgánicos.[31,32] También tiene importancia para la detección de lesiones específicas en combinación con el ensayo de hibridación fluorescente *in situ*.[33]

Protozoos

Los protozoos son componentes importantes de los ecosistemas acuáticos, o ambientes húmedos, donde desempeñan un papel crucial en el funcionamiento de los sistemas tróficos.[34] Con las

ventajas de su gran diversidad, ciclo de vida corto, elevado grado de reproducibilidad y respuestas rápidas a las perturbaciones ambientales, cumplen todos los requisitos para ser utilizados como organismos de ensayo en evaluaciones ambientales.[35,36] Varios estudios confirman que los protozoos son organismos de ensayo idóneos para los estudios de ecotoxicología empleando el EC. Un ejemplo ilustrativo lo constituyen los estudios realizados en *Blepharisma japonicum*, un protozoo ciliado que posee un pigmento fotoreceptor que, bajo la acción de la luz, genera especies reactivas del oxígeno que inducen fragmentación del ADN detectable por este ensayo.[37] Otro ejemplo es el estudio del daño al ADN inducido por rayos *gamma* mediante ensayo cometa neutral en *Cryptosporidium parvum*, un protozoo parásito intracelular obligado.[38] Los protozoos son considerados como indicadores ideales de alerta temprana en el deterioro de los ecosistemas acuáticos.[39]

Pequeños crustáceos (Artemia)

Muchas especies de moluscos, crustáceos y poliquetos, forman parte de la cadena alimenticia humana, por lo que resulta importante contar con procedimientos que permitan determinar el efecto de los agentes mutagénicos y carcinogénicos del ambiente sobre estos organismos. La mayor parte de ellos tienen la capacidad para transformar esos agentes a metabolitos biológicamente activos y de acumular las toxinas en células y tejidos en concentraciones superiores a las encontradas en el medio.[21] El ambiente marino constituye un depósito de muchos compuestos químicos naturales y antropogénicos potencialmente tóxicos, por lo que el monitoreo de sus efectos sobre el ambiente y las especies que en él habitan es de primordial importancia. Las especies del género Artemia forman parte de la cadena trófica y contribuyen de manera relevante a la alimentación de especies importantes de consumo humano.[40,41] El género Artemia ha sido empleado como modelo en estudios de ecología, fisiología, ecotoxicología, acuacultura y genética, debido a su amplia distribución geográfica y adaptabilidad a condiciones extremas de ambientes hipersalinos.[42,43] El empleo del EC en diferentes especies de artemias, ha sido desarrollado implementando protocolos apropiados para la obtención de suspensiones celulares.[44] Un ejemplo que demuestra la efectividad del EC es el estudio realizado en diferentes especies de artemias con diferentes estrategias reproductivas (*Artemia franciscana*e, *Artemia parthenogenetica*), las cuales fueron expuestas a etilmetanosulfonato.[45] La comprensión de los efectos tóxicos sobre estos organismos es importante para la predicción de la contaminación del ambiente marino.

Insectos

Drosophila melanogaster es el organismo modelo por excelencia para estudios de genética, biología del desarrollo e investigaciones toxicológicas.[46,47] El Centro Europeo para la Validación de Métodos Alternativos (CEVMA), ha impulsado los estudios en este organismo.[48] La validación y aplicación de *Drosophila melanogaster* como un modelo *in vivo* para la detección de rupturas de cadenas por el EC sugiere la posibilidad de su empleo para la evaluación *in vivo* de contaminantes químicos ambientales que pueden inducir roturas de cadenas.[49] Los ensayos realizados en hemocitos de *Drosophila melanogaster* han permitido evaluar el potencial genotóxico de dos compuestos de níquel ($NiCl_2$, $NiSO_4$).[50] Otro estudio utilizó células intestinales, para evaluar la genotoxicidad del benceno, el tolueno y el xileno ampliamente utilizados para fines industriales y domésticos[51]

Lombriz de tierra

En la evaluación de los efectos agudos y crónicos de los compuestos tóxicos en el suelo, se han empleado, además de las plantas, diferentes especies de animales, entre ellas, los anélidos, como la lombriz de tierra.[52] Son invertebrados de importancia ecológica y útiles en estudios ecotoxicológicos. Las especies de lombriz comúnmente utilizadas en ecotoxicología son *Eisenia*

fetida y *Eisenia andrei,* aunque se han empleado otras como *Amynthas diffringens, Aporrectodea caliginosa, Dendrodrilus rubidus* y *Microchaetus benhami* en estudios de sensibilidad entre especies.[53] Han sido empleadas para monitorear la toxicidad que provocan xenobióticos tales como los hidrocarburos policíclicos aromáticos en suelos contaminados,[54] plaguicidas como el imidacloprid y el RH-5849,[55] algunos metales pesados como el níquel y el Cr (VI) empleando celomocitos de *Eisenia fétida*.[56-58] Las lombrices de tierra desempeñan un papel importante en las cadenas alimenticias terrestres como un alimento importante en las dietas de muchos animales, en la descomposición de la materia orgánica, el ciclo de nutrientes y en la fertilidad de los suelos., La contaminación del suelo en dependencia de su magnitud puede ser perjudicial para la población de lombrices por lo que es necesario comprender sus efectos adversos sobre ellas para predecir posibles efectos de la contaminación en la cadena alimentaria.[59,60]

Moluscos

Los estuarios y el medio ambiente costero, son depósitos geográficos naturales de barro y arena. Ellos tienen una gran susceptibilidad a la contaminación con productos químicos y petróleo procedentes de los desagües urbanos, desechos industriales, cisternas y tuberías de descarga.[61] Los mejillones (*Mytilus* sp.), son especies centinelas de los estuarios[62] ampliamente utilizados como biomarcadores debido a su naturaleza sésil, alta capacidad de filtrado y de bioacumulación de contaminantes ambientales. Su distribución ubicua, y eficiente capacidad de reciclaje de contaminantes en los ecosistemas de estuario, hacen valiosa esta especie para investigaciones ambientales y ecotoxicológicas.[63] La sensibilidad a los genotóxicos y al estrés ambiental en exposiciones agudas y crónicas de contaminantes ambientales se ha demostrado en hemocitos, glándulas digestivas y células de branquias.[64,65] Para los estudios de biomonitoreo, la medición del daño al ADN es empleada como un marcador en la evaluación de mezclas de contaminantes.[66,67] Diversos estudios han utilizado la especie *Mytilus edulis* para revelar la contaminación marina por estireno, cadmio, cromo (VI) y otros compuestos.[68-71] La exposición experimental de mejillones en jaulas, durante tres semanas a las aguas de ríos contaminados de Croacia, evidenció un aumento del daño al ADN en hemocitos de esta especie.[72] Un estudio en células de branquias de mejillones colectados en una zona de la costa de Dinamarca demostró, mediante el EC, un mayor índice de rotura de cadenas.[73] Otro estudio, que utilizó embriones de ostras expuestos al benzo[a]pireno demostró igualmente un incremento de las roturas de cadena de ADN.[74] La exposición a fulerenos y fluoranteno, mostró mediante el EC una mayor frecuencia de roturas de cadena en hemocitos de mejillones.[75] También existen evidencias de que estos organismos marinos pueden ser susceptibles al daño físico por radiación ionizante en fuentes contaminadas.[76]

Peces

Los peces han sido utilizados frecuentemente como organismos centinelas en estudios de ecotoxicología.[77,78] Ellos desempeñan roles importantes en la cadena trófica, acumulan sustancias tóxicas y responden a las bajas concentraciones de mutágenos. Su empleo como biomarcadores de contaminación, se basa en su susceptibilidad a cambios ambientales, de lo que se desprende su importancia para la evaluación del riesgo potencial de contaminación por nuevos compuestos químicos en ambientes acuáticos.[79] El registro de los parámetros genotóxicos en peces se encuentra entre los más valiosos marcadores biológicos para la evaluación del riesgo ambiental.[80] El EC ha demostrado una amplia aplicación para evaluar el daño al ADN en peces expuestos a diversos xenobióticos del medio acuático.[10,81,82] Se han realizado estudios en diferentes especies de peces, como por ejemplo *Channa punctatus* en la que se evaluó la toxicidad del malatión,[82] en *Vibrio fischeri* en la que se analizó la contaminación de aguas de un río y un lago situado cerca de un hospital[83] y en larvas de *Gasterosteus aculeatus* en las que se determinaron los efectos crónicos del herbicida atracina.[84]

Anfibios

Los anfibios constituyen otro grupo de organismos en el que el EC tiene gran aplicación debido a la permeabilidad de su piel en comparación con otras especies.[85,86] Pueden absorber fácilmente las sustancias disueltas en el agua, entre ellas, los contaminantes petroquímicos.[87] El EC ha sido adaptado a diferentes especies de anfibios, entre ellos *Anaxyrus americanas,*[88] *Pelophylax nigromaculata,*[89] *Pelophylax lessonae,*[90] *Eleutherodactylus johnstonei.*[91] Se ha utilizado para la evaluación de herbicidas,[92] pesticidas,[93] y aguas de ríos contaminados.[94] El impacto de estos productos sobre los anfibios es preocupante dada su gran sensibilidad a los plaguicidas, a los cambios del medio ambiente[95] y la rápida disminución de sus poblaciones que se observa a nivel mundial en los últimos tiempos.[96]

Mamíferos silvestres

Los mamíferos silvestres son organismos muy utilizados en estudios de ecotoxicología terrestre. Ellos asimilan contamiantes a lo largo del tiempo y poseen una serie de características específicas por las cuales resultan especialmente útiles como bioindicadores.[97] Entre ellos se pueden encontrar especies de roedores de las familias *Ctenomyidae, Geomyidae, Soricidae* y liebres de la familia *leporidae*. Estos organismos acumulan residuos de productos agrotóxicos a partir de sus alimentos los cuales le causan efectos tóxicos y lesiones en el material genético.[98] El EC se realizó fácilmente en estos organismos fundamentalmente por su rápida reproductibilidad y el tamaño de sus poblaciones. Por ejemplo, los estudios realizados en roedores *Ctenomys torquatus* expuestos a carbón mineral de Río Grande do Sul, Brazil. En ellos, se realizó el EC en sangre periférica el cual demostro que el carbón induce lesiones en el ADN de estos roedores.[99] Otro ejemplo ilustrativo en poblaciones silvestres de *Mus musculus*, demuestra que los animales de este tipo que viven alrededor de las zonas mineras de carbón tienen una mayor probabilidad de sufrir daños en el ADN.[100] Otros mamíferos también son utilizados para la realización de este ensayo aunque en menor proporción. Entre ellos, se tiene el estudio en delfines de la laguna Indian River en Florida, Estados Unidos. En ellos, se realizó el EC en linfocitos de sangre y se encontro en algunos individuos mayor frecuencia de rupturas de hebras de ADN debido a un aumento de la concentración de agentes genotóxicos en las proximidades de este sitio.[101]

CONCLUSIONES

El Ensayo Cometa ha sido aplicado en múltiples especies y ha demostrado ser un método sensible para la detección de la genotoxicidad de productos químicos y mezclas complejas así como en el biomonitoreo ambiental. Su aplicación tanto *in vitro* como *in vivo* en organismos modelo amplía su empleo en estudios de ecotoxicología. La variedad de hábitats de los organismos empleados en el ensayo y su elección adecuada permite una correcta evaluación del daño a nivel genético en especies que viven en ambientes contaminados. Por ejemplo, las lombrices de tierra son organismos útiles para los estudios ecotoxicológicos en suelos contaminados. Moluscos, anfibios y peces pueden emplearse para la evaluación de genotoxicidad *in situ* de aguas contaminadas y estos últimos son eficaces en la detección de genotoxicidad *in vitro* de sedimentos de ríos. Por otra parte, los mamíferos silvestres como organismos superiores que asimilan contaminantes a lo largo del tiempo, son una buena elección en estudios de ecosistemas marinos y terrestres muy contaminados. En conclusión, los múltiples estudios que emplean este ensayo demuestran su sensibilidad, rapidez y eficacia, lo que a su vez, lo hacen recomendable para estudios de ecotoxicología terrestre y marina.

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ANEXO 2

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Allium cepa and *Tradescantia pallida* bioassays to evaluate effects of the insecticide imidacloprid

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HIGHLIGHTS

- The genotoxic potential of imidacloprid were studied.
- Imidacloprid had a genotoxic effect on the two organisms tested (*Tradescantia pallida* and *Allium cepa*).
- Imidacloprid has an environmental risk.
- Imidacloprid genotoxic risks should be taken into consideration when using this insecticide in agriculture.

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ABSTRACT

The indiscriminate use of pesticides has become a serious environmental concern. Of them, imidacloprid (IMI) is one of the most widely used worldwide. In 2010 in Brazil, 1.934 tonnes of IMI were sold and mainly used for sugarcane crops. Several studies have examined the toxicity of IMI as well as its possible ecological effects. However, few studies have examined its toxicity at the genetic level. This is one of the biggest challenges for the scientific community, which is concerned about the impacts of these contaminants on the environment and human health. In this study, we evaluated the effects of IMI above the genetic material in *Allium cepa* and *Tradescantia pallida* following exposure to different concentrations of this insecticide. The results demonstrated that the concentrations tested induced chromosomal alterations and increased the frequency of micronuclei. Therefore, IMI in these concentrations was genotoxic to the tested organisms. These factors should be taken into account when applying this pesticide.

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1. Introduction

Imidacloprid (IMI; 1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine) belongs to a class of chemical compounds called neonicotinoids that act on the central nervous system of insects. It is one of the top selling and most widely used insecticides in the world (Nauen and Bretschneider, 2002) for the purpose of controlling sucking insects, chewing insects, and termites affecting crops. It is applied directly to the seeds, soil and cultures and can be used in topical treatments to control fleas on pets (Mullins, 1999). In Brazil, it is commonly used in sugarcane, citrus, cotton, and coffee crops (AlamosBrasil, 2008). In 2010, companies reported sales of 1.934 tonnes of IMI to the Brazilian Institute of Environment and Renewable Natural Resources. The aerial application of this insecticide has been associated with the death of bees in different regions of the country, which has led to its prohibition. Currently open to re-evaluation, the aerial application of pesticides is prohibited during flowering and when bees are foraging on any type of crop due to its negative effects (IBAMA, 2012).

Insecticides can have toxic effects on plants and animals due to the presence of residues in the soil, water, and air. IMI is biotransformed into several metabolites that degrade completely into carbon dioxide (Bacey, 2000), and field studies have shown that it can persist in soil, with a half-life of 27–229 days (Meister, 1994). In addition to environmental contamination, its residues

Abbreviations: IMI, Imidacloprid; USEPA, United States Environmental Protection Agency; MI, Mitotic index; CAI, Chromosomal aberration index; CAs, Chromosomal aberrations; MN, Micronuclei; CB, Chromosome breaks; MMS, Methyl methanesulfonate; CHO, Chinese Hamster Ovary; RLS, Resonance Light Scattering.

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can enter the food chain, potentially reaching humans and posing health risks (Edwards, 1973). There have been several toxicity studies of IMI in attempts to obtain its registration and commercialisation; among them, acute toxicity studies in mice and rats have indicated that this insecticide is moderately toxic (Kidd and James, 1991; Meister, 1994), classifying it as a Class III compound according to its median lethal dose (Gallo et al., 2002). Genotoxicity studies have reported the possible presence of alterations in the chromosomes of human lymphocytes and CHO cells following exposure to IMI (Federal Register, 1995). However, carcinogenicity studies led to its classification as a "Group E" carcinogen with minimal risk to human health by USEPA (1995).

In countries with an agriculture-based economy, the use of a wide variety of pesticides is a major concern, and therefore, toxicological studies are extremely important. The use of live organisms (bioindicators) can reveal the presence of stressors from environmental pollutants (Carneiro and Takayanagui, 2009) and monitor their negative effects. Several studies have been conducted with plants, aquatic organisms, amphibians, insects, annelids, birds, and rodents demonstrating the toxicity of different pesticides used in agriculture (Iannacone et al., 2007; Amaro, 2009; Dittbrenner et al., 2011). Studies at the genetic level that indicate changes in the genotypes and phenotypes of individuals are also important because these alterations can cause ecological disturbances at the population and community levels (Shugart and Theodorakis, 1996). Many of the pesticides used to control pests in different areas, despite their benefits, may potentially induce genetic alterations in plants and animals (Bouhafs et al., 2009; Cavas, 2011; Dante, 2011).

Higher plants are used worldwide in environmental biomonitoring. They are considered to be excellent genetic models for detecting environmental mutagens (Haywood et al., 2007; Andrioli et al., 2012; Herrero et al., 2012). Among plant species, *Allium cepa* has been used to evaluate DNA damage, such as CAs and disturbances in the mitotic cycle. It has also been widely used to evaluate xenobiotics and is an important tool for environmental biomonitoring (Leme and Marin-Morales, 2009). *Tradescantia pallida*, which is known for being highly sensitive to mutagens, is also suitable for monitoring studies. The MN test using this species is an important tool in environmental studies (air pollution, chemical contaminants in the water and soil). When exposed to genotoxic and mutagenic agents, its reproductive cells exhibit nuclear fragments (MN) resulting from chromosomal breakage or loss, indicating genotoxic damage (Carneiro and Takayanagui, 2009; Mielli, 2009).

This study aimed to evaluate the effects of IMI above the genetic material in the organisms *A. cepa* and *T. pallida* following exposure to different concentrations that are typically used in sugarcane crops, which were applied directly to the soil.

2. Materials and methods

2.1. Test compound – IMI

The compound used in this study was IMI (1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine) (CAS N°138261-41-3, molecular formula of $C_9H_{10}ClN_5O_2$), which was obtained from Bayer Crop Science, Agricultural Experiment Station-SP, Lot EDE 0036241.

2.2. Treatment solution

The insecticide IMI was diluted in distilled water, which was based on the maximum concentration of the commercial product applied to the soil (40 mg m^{-2}), half of the recommended concentration to simulate natural dilution (20 mg m^{-2}), and twice the concentration (80 mg m^{-2}) to simulate indiscriminate use.

2.3. Biological materials

2.3.1. A. cepa

The bioassays were conducted with *A. cepa* seeds (Liliaceae) of the Baia Periforme variety (Isla Sementes© brands, Lot 32443-S2) to minimise variations in treatment response. The seeds were stored in the dark at 6–10 °C until use.

2.3.2. T. pallida

Inflorescences of *T. pallida* were obtained from standardised garden beds, which were maintained at the Experimental Garden of the Institute of Biosciences, UNESP, Rio Claro Campus, São Paulo, Brazil.

2.4. Bioassays

2.4.1. A. cepa assay

To evaluate the toxic, cytotoxic, genotoxic, and mutagenic potentials of the different concentrations of IMI, 100 *A. cepa* seeds were used in each bioassay according to a modified version of Grant's protocol (1982). The seeds were allowed to germinate at a temperature of 23 °C in petri dishes lined with paper filters moistened with IMI. The positive control consisted of 0.019 ppm of the aneugenic herbicide Trifluralin® (CAS N°1582-09-8) (Fernandes et al., 2007). The negative control consisted of seeds allowed to germinate in ultrapure water. All bioassays were conducted in duplicate. The seeds remained in petri dishes for 96 h. Those that did not germinate were quantified. Root tips were collected and fixed for slide preparation and cytogenetic analyses. The materials were fixed in Carnoy's solution (3:1 ethanol:glacial acetic acid) for 6 h and then transferred to fresh Carnoy's solution and maintained in a refrigerator until use. For the cytogenetic analyses, the roots were stained by the Feulgen reaction (Mello and Vidal, 1978), which consisted of an acid hydrolysis with 1 N HCl at 60 °C for 9 min followed by rinsing with distilled water. The materials were then immersed in Schiff reagent for 2 h. For the slide preparation, 2% acetic carmine was used. The root meristems were gently pressed between the slides and coverslips. The latter was removed with liquid nitrogen, and the slides were mounted with Permount for examination. On each slide, 1000 meristem cells of *A. cepa* were examined (totalling approximately 5000 cells per treatment) under a light microscope.

Toxicity was evaluated based on the seed germination index, which was calculated as the ratio of the number of germinated seeds to the total seeds allowed to germinate.

Cytotoxicity was assessed based on the quantification of morphological alterations indicating cell death and the MI, which was characterised by the total number of dividing cells in the cell cycle and was calculated by the formula MI = (number of dividing cells/total number of observed cells) × 100.

Genotoxicity was evaluated based the CAI, which was calculated by the formula CAI = (number of cells with CA/total number of observed cells) × 100.

The results obtained for all concentrations were compared with those of the negative control using the non-parametric Kruskal–Wallis test with significance set at 0.05; the analyses were performed using the BioEstat 5.0 software.

2.4.1.1. Evaluation of MN in cells from the F_1 region of A. cepa. To examine damage fixation in the meristematic cells, the MN were quantified in 5000 cells from the F_1 region for each treatment. The results obtained for all samples were compared with those of the negative control using the Kruskal–Wallis test with

significance set at 0.05, and these analyses were performed with BioEstat 5.0 software.

2.4.2. T. pallida assay

The *T. pallid* MN test was developed according to Ma et al. (1994) protocol, with modifications. To evaluate genotoxicity, ten young inflorescences (all with closed flower buds) of approximately 6–8 cm in length were collected from standardised flower beds and placed in beakers for 8 h under constant aeration with IMI at one of the three concentrations. After this period of exposure, the inflorescences were transferred to beakers with ultrapure water, where they remained for 24 h for possible recovery. The same protocol was followed for the negative (exposure of inflorescences to ultrapure water) and positive (exposure of inflorescences to 10 $\mu L\ mL^{-1}$ of MMS) controls. After this period, four clusters of buds were selected and fixed with Carnoy's solution for 24 h. Then, the medium-sized bud was used to prepare the slides. The material was placed on a slide and macerated with a razor blade to remove the remnants of inflorescence. To facilitate the procedure and demonstrate the presence of micronucleated tetrads, a drop of 2% acetic carmine (Sisenando et al., 2009) was added to the material and coverslipped. The slide was then quickly passed over a flame. Ten slides were examined per treatment under a light microscope to examine the presence of MN, and a total of 3000 tetrads were observed per treatment. The results obtained for all samples were compared with those of the negative control using the Kruskal–Wallis test with significance set to 0.05, and these analyses were performed with the BioEstat 5.0 software.

3. Results

The results from the *A. cepa* assay, in which the cells were exposed to the three concentrations of IMI and the positive and negative controls, are shown below in Table 1. The MI was analysed, which represented the number of dividing cells, and no significant differences were observed when comparing the treatments with the negative control. The CAs and nuclear abnormalities observed in the present study were visualised at all stages of the cell cycle, including interphase, prophase, metaphase, anaphase, and telophase. Several types of aberrations within different cell division stages were considered (chromosome fragments, losses and bridges, vagrant chromosomes, delays, adherence and other aberrations) (Leme and Marin-Morales, 2009). However, they were classified into just one category to evaluate the CAI as a single endpoint. The most frequent alterations observed in this study were binucleated cells, metaphases with chromosome loss, anaphases with chromosome bridges, and anaphases with chromosome loss.

Table 1
Mean and standard deviation of the mitotic, chromosomal aberrations index in 5000 meristematic cells of *A. cepa* and MN in cells of the F_1 region after exposure to ultrapure water (negative control), trifluralin (positive control) and three concentrations of IMI.

Groups	Parameters analysed/5000 cells		
	MI	CAI	MN (F_1 region)
NC (H_2O)	10.307 ± 1.03	0.115 ± 0.05	0.3 ± 0.15
IMI (20 $mg\ m^{-2}$)	10.341 ± 1.48	1.302 ± 0.28*	0.4 ± 0.16
IMI (40 $mg\ m^{-2}$)	7.71 ± 1.37	1.48 ± 0.18*	0.8 ± 0.38
IMI (80 $mg\ m^{-2}$)	11.658 ± 1.60	1.584 ± 0.24*	1.8 ± 0.35*
TFA (0.019 ppm)	9.768 ± 1.19	1.873 ± 0.23*	4.4 ± 0.93*

NC: negative control; TFA: trifluralin-positive control; IMI: imidacloprid; MI: mitotic index; CAI: chromosomal aberration index; MN: micronuclei in cells of the region F_1.
* $p < 0.05$. Values statistically significant, compared to negative control with the Kruskal–Wallis test.

Anaphases with chromosome breakages and meristematic cells in interphase with MN were also observed. All concentrations of IMI induced genotoxic effects (Table 1) when compared with the negative control ($p < 0.05$), based on the CAI. The presence of MN in the F_1 region at the highest concentration of IMI (Table 1) was statistically significant compared with the negative control ($p < 0.05$), suggesting the permanence of the damage.

In the *T. pallida* test, significant increases in the frequencies of MN (Fig. 1) were observed in the tetrads at the three concentrations of IMI compared with the negative control ($p < 0.05$).

4. Discussion

The intensive use of pesticides in agriculture is associated with a wide range of potential environmental risks. Despite their benefits, they can also induce genetic alterations in plants and animals. Furthermore, the indiscriminate use of these chemicals can cause poisoning and environmental contamination. To evaluate the possible cytotoxic, genotoxic and mutagenic effects of these substances, organisms that are easily obtainable and sensitive to pesticide exposure may be used as indicators in association with different biomarkers (Silva et al., 2003).

The neonicotinoid IMI is one of the most frequently used pesticides. Thus, the assessment of the effects of different concentrations of IMI on genetic material from two test organisms, as conducted in the present study, is extremely important to provide information on this widely used insecticide.

The *A. cepa* assay is commonly used in studies on pesticides, such as the herbicide trifluralin, which is capable of interfering with cell division (Fernandes et al., 2007, 2009), and chlorfenvinphos and fenbuconazole (Türkoglu, 2012), which are two compounds that possess genotoxic effects. Studies on organophosphate pesticides, such as Br-containing oxaphosphole, have also been conducted. These chemical compounds can affect proliferating cells and cause disturbances in genetic material (Kalcheva et al., 2009). Azzurro®, which is a fungicide that is widely used in agricultural and industrial applications (Andrioli et al., 2012), has also been evaluated using the *A. cepa* test.

MI is a parameter that allows for the estimation of the frequency of cellular division, which is used to reliably identify the presence of cytotoxic pollutants in the environment (Fiskesjö,

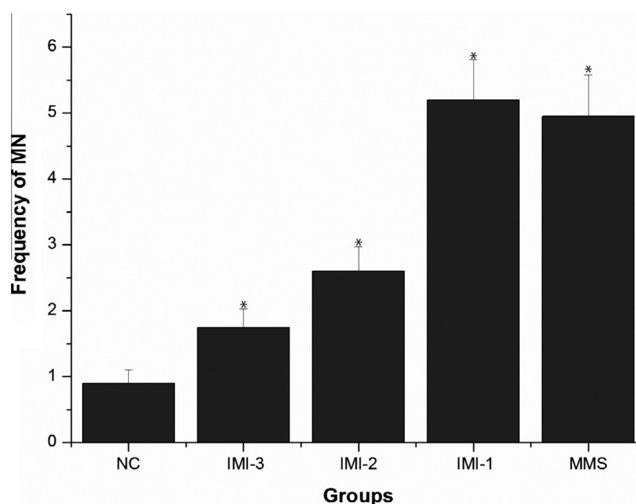

Fig. 1. Frequency of MN in 3000 tetrads per experimental group (mean ± SD). MMS (10 $\mu L\ mL^{-1}$): methyl methanesulfonate (positive control); NC: negative control (distilled water); IMI-1: imidacloprid (80 $mg\ m^{-2}$); IMI-2: imidacloprid (40 $mg\ m^{-2}$); IMI-3: imidacloprid (20 $mg\ m^{-2}$). * $p < 0.05$, significant differences compared with the negative control, as shown by the Kruskal–Wallis test.

1985). This study did not reveal a significant reduction in the MI at the evaluated IMI concentrations. The results obtained for the different concentrations of IMI revealed a significant dose-dependent increase in the frequency of CAs in the *A. cepa* cells. Similar effects have been observed in studies of the fungicides Azzurro® (Andrioli et al., 2012) and flusilazole (Ozakca and Silah, 2013). It has been reported that DNA breakages and the inhibition of DNA synthesis and replication are the main causes of structural chromosomal alterations. They can be spontaneous or induced by physical and chemical agents (Obe et al., 1992; Bryant, 2004). CA, such as bridges and breaks, suggest clastogenic activity, while chromosome losses indicate aneugenic effects (Leme and Marin-Morales, 2009). Chromosome bridges result from chromosome and/or chromatid breakage and fusion, whereas laggard chromosomes increase the risk of aneuploidy (Grover and Kaur, 1999). Generally, cytotoxic agents cause disturbances of the spindle, resulting in C-mitosis, C-anaphase and multipolarity effects. It has also been demonstrated that some CAs may occur through non-direct genotoxic effects. Some damage induced by metals can inhibit DNA repair mechanisms by competing with certain ions, such as those that are essential for DNA polymerases (Cebulska-Wasilewska et al., 2005). Indeed, some CAs were induced by DNA breaks, but repair mechanisms intervened to repair these anomalies and maintain genomic integrity (Vodicka et al., 2004). Moreover, some pesticides may cause indirect genotoxic effects, such as the decreased levels of DNA repair that have been observed following glyphosate exposure (Cavas and Konen, 2007). Treatments with some chemicals have been shown to induce the deregulation of cell cycle events (Zabka et al., 2012) and the activation of surveillance mechanisms to stop the cell cycle, allowing time for the completion of a particular cell cycle event. These checkpoints are essential for cell survival under adverse conditions, and the alteration of one of these checkpoints by a xenobiotic agent could disorganise all division processes and lead to aberrant mitosis (Rybaczek et al., 2007). Anaphase with chromosome breaks and MN cells were the most commonly observed abnormalities at the three concentrations of IMI evaluated. Clastogenic and aneugenic activities are inferred by the presence of MN. These structures can be formed from acentric fragments as a result of a clastogenic actions or the loss of an entire chromosome due to aneugenic activities (Fenech, 2000). In this study, the presence of MN in cells from the F_1 region was observed only in the group exposed to the highest concentration of IMI, demonstrating its mutagenic potential. Similar results have been previously reported for other pesticides (Ventura et al., 2008; Cavas, 2011). The damage induced in meristematic cells exposed to the two lowest concentrations may have been repaired because no significant differences were found in the frequencies of MN in the cells from the F_1 region.

Another plant used in the present study was *T. pallida*. However, few studies have used this species to evaluate the effects of pesticides (Raino et al., 2010) because it is most commonly used to evaluate environmental samples (Savóia et al., 2009; Crispim et al., 2012; Pereira et al., 2013). The results involving *T. pallida* indicate significant dose-dependent increases in the frequencies of MN in the groups exposed to the three IMI concentrations. The results obtained for *T. pallida* support those for *A. cepa*.

Field studies have shown that IMI can remain in the soil (Meister, 1994), where it is biotransformed into various metabolites until being completely degraded into carbon dioxide (Bacey, 2000). Its presence can be toxic to organisms in the ecosystem, such as bees (de Almeida-Rossi et al. (2013)), earthworms (Dittbrenner et al., 2011) and amphipods (Maley et al., 2012). Studies of its possible genotoxic effects on human lymphocytes have also been conducted. A weak clastogenic activity (DNA breaks) was observed in the presence of metabolic activation. In CHO cells, a significant increase in sister chromatid exchange was observed at concentrations of 2 and 3 mg mL^{-1} with metabolic activation (Cordone and Durkin, 2005). Another study of the genotoxicity of IMI reported the interaction between DNA and this pesticide as revealed by RLS. The mechanism underlying this interaction is still unknown. The RLS of DNA was inactivated by the addition of IMI to an aqueous solution with a pH value of 2.10 (Shicong et al., 2009). The data obtained in this investigation confirm the USEPA classification of both pesticides as possible human carcinogens.

5. Conclusions

Results here demonstrated that IMI had a genotoxic effect on the two organisms tested at the three concentrations assessed; therefore, it poses a potential environmental risk. Because the use of pesticides cannot be avoided, lower doses would be beneficial in addition to the development of less toxic substances with similar effects, which could lead to healthier environments for future generations.

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ANEXO 3

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Genotoxic Potential of the Insecticide Imidacloprid in a Non-Target Organism (*Oreochromis niloticus*-Pisces)*

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Abstract

The indiscriminate use of pesticides has become a serious environmental concern. Insecticide imidacloprid (IMI) is the second most widely used pesticides worldwide. In 2010, 1.934 tons of IMI were sold in Brazil, mostly to be used in sugarcane crops. Several studies have detected its presence in the aquatic environment constituting a risk to non-target organisms. The aquatic animals are organisms used for environmental biomonitoring worldwide. They are considered excellent genetic models to detect environmental mutagens. Among animal species, the fish *Oreochromis niloticus*, commonly known as Nile tilapia, has been used to evaluate DNA damage. The present study therefore evaluated the effect of IMI on the genetic material of *Oreochromis niloticus* (Pisces) erythrocytes exposed to different concentration (250; 125 and 62.5 μg/L) of IMI used in growing sugarcane. The effect of the IMI was measured using the comet assay and the micronucleus (MN) test, assays that detected genotoxic damage. The results in the comet assay demonstrated that the concentrations tested induced primary damage to DNA. They also proved the occurrence of MN and nuclear abnormalities at the higher concentration used in the micronuclei and other nuclear abnormalities test. The insecticide IMI induced primary DNA damage at all concentrations and

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damage at the chromosomal level at the highest concentration tested. The results of this study show the potential risk of IMI in a non-target organism.

Keywords

Pesticide, Genotoxicity, DNA Damage, Comet Assay, Micronucleus Test

1. Introduction

The use of sugarcane as a biofuel has expanded in the last decade and Brazil becomes the largest producer in the world [1]. The increase in sugarcane crops is accompanied by the increase of pesticide consumption. The imidacloprid (IMI; 1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine) belongs to a class of chemical compounds called neonicotinoids that act on the central nervous system of insects and it is the second most used pesticide in the world [2]. In 2010, companies reported sales of 1.934 tons of IMI to the Brazilian Institute of Environment and Renewable Natural Resources, principally for use in sugarcane.

The IMI can persist in soil, with a half-life (28 - 1250 days) highly variable which varies greatly among soil type and other factors [3]. Also, depending on rainfall and soil conditions, 2.4% to nearly 80% of the mass of neonicotinoids (including IMI) could make their way into water bodies [4]. Due to its presence in various environments, inhabited by large numbers of organisms, toxicological studies are extremely important. Thus, the use of living organisms (bioindicator), capable of somehow indicating the presence of stresses generated by environmental pollutants [5] [6], is one way to monitor the negative effects in the environment. Due to the presence of significant levels that have been detected in water, it is very important to conduct studies on the effects of IMI on aquatic organisms as bioindicators. Fish are widely used because of capacity to accumulate contaminants and can show physiological, biochemical, histological or differentiated cell response [7]. These organisms may indicate variations in tolerance to environmental conditions created by the use of pesticides, including genetic change, which makes them excellent indicators with a high application for monitoring environmental genotoxicity [8]. Among the most commonly used species of fish is *O. niloticus*, known as Nile tilapia, considered optimal in laboratory studies for its multiple advantages such as a high rate of growth and reproduction, resistance and tolerance in laboratory conditions and adaptation in commercial alimentation [9].

There are many studies in target and non-target organisms, including mammals, birds, fish, insects, crustacean, molluscs and annelids, showing the toxicity of various neonicotinoids used in agriculture [3]. Furthermore, genotoxicology studies using biomarkers of DNA damage are very important. The tests most used to assess DNA damage in fish exposed to pesticides and other environmental contaminants are the comet and micronucleus tests, excellent tool in genotoxicology, both used in this study.

The alkaline single-cell gel electrophoresis assay, better known as the comet assay, detects strand breaks, alkali-labile sites, and delayed-repair sites as initial damage [9]. The micronucleus (MN) test associated with nuclear abnormalities (NAs) has been used for more than 30 years and also is one of the most promising trials in ecotoxicology for damage detection at the chromosomal level [10] [11]. These tests of genotoxicity are recognized due to their robustness, sensitivity and statistical power to evaluate DNA damage.

In this context, the present study provides more information about the effects of the used of this insecticide in non-target organisms, providing important data on ecotoxicological consequences.

2. Material and Methods

2.1. Test Compound-IMI

The compound used in this study was IMI (1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine; CAS N˚138261-41-3; molecular formula $C_9H_{10}ClN_5O_2$), Agricultural Experiment Station-SP, Lot EDE 0036241.

2.2. Treatment Solution

The Concentrations of IMI used in the present study for the exposure of *O. niloticus* were determined through

concentration used in growing sugarcane [12] and simulating natural insecticide dilution into water resources [3]. The insecticide IMI was applied in water at three concentrations C1 = 250 µg/L twice the concentration to simulate indiscriminate use, C2 = 125 µg/L based on the maximum concentration of the commercial product applied in sugarcane and C3 = 62.5 µg/L, half of the recommended concentration to simulate natural dilution.

2.3. Biological Material

The test organism used in the experiment was *O. niloticus* (Perciformes, Cichlidae) (n = 50), popularly known as Nile tilapia. Individuals with a mean size of 12 - 15 cm were analysed to avoid intra-specific differences related to size, age (two months) and average body weight (30 ± 2 g). The individuals analysed were reared on fish culture farms and kept in the Experimental Garden of the Institute of Biosciences, UNESP (São Paulo State University), Rio Claro, São Paulo, Brazil. They were brought to the laboratory and acclimated in aerated aquariums (size: length = 45 cm, height = 25 cm, width = 20 cm) for one week at a mean temperature of 23˚C, in tap water (pH = 8.3, Temp= 20˚C ± 2˚C) and 14 hours light/dark cycle.

2.4. Bioassay

Ten aquariums were used in the experiment, two for negative control (NC) (fresh water), two in which animals received an intraperitoneal (i.p.) injection the clastogenic agent cyclophosphamide (20 mg/mL) (in sterile water) (30 mL of cyclophosphamide/50 g of fish) for the positive control (PC) [13] and the remaining six were exposed to the three IMI concentrations (250; 125 and 62.5 µg/L). All treatments consisted of two replicates with five organisms, each one in a 40 L aquarium, which was aerated during the 96h exposure period [13] [14] and no food was supplied to the fish during the experiment. Approximately 0.3 cm^3 of whole blood was taken from each fish by heart puncture using heparinised syringes. The collected blood was used for the comet assay and the MN and NA test. The study was approved by "The Ethics Committee on Animal Use", UNESP, filed with the number 8937.

2.5. Comet Assay

The alkaline comet assay was performed as described by Collins (2004) with the modifications suggested by Caffetti *et al.* (2008) [15] [16]. The blood was obtained from the fish as described above and 3 µL aliquots were diluted in 1 mL of PBS (137 mM NaCl, 2.68 mM KCl, 8 mM HNa_2PO_4; H_2KPO_4 1.47 mM) to obtain the cell suspension. Microscope slide coded were coated with 1% (v/v) standard agarose and 10 µL of the diluted blood with 120 µl the 0.5% (v/v) low melting point agarose were applied to the slides at 37˚C. After, cover slips were placed on the slides for 10 min at 4˚C to ensure the formation of the microgel. The slides were placed in lysis buffer (1 mL of Triton X-100, 10 mL of DMSO, and 89 mL of solution plus, which included 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, ~8.0 g of NaOH and 10 mL of 1% (v/v) sodium lauryl sarcosinate), pH 10, in a refrigerator for 1 h. After lysis, the slides were incubated in 300 mM NaOH + 1 mM EDTA buffer (pH > 13) for 20 min to denature the DNA and then submitted to electrophoresis at 39 V and 300 mA for 20 min; field strength 0.8 V/cm. The slides were then neutralised with PBS for 15 min and fixed in absolute ethanol for 10 min. The slides were stained with Gel Red Nucleic Acid (Gel Stain Biotum 10,000× water) and analysed (blindly) under an Olympus BX60 fluorescence microscope equipped with filter excitation: k = 420 - 490 nm, emission barrier: k = 520 nm) and a 40× objective lens.

For each fish, 100 nucleoids were analysed per blood sample. The nucleoids were visually classified according to fragment migration as undamaged (class 0), slightly damaged (class 1), more damaged (class 2) and highly damaged (class 3) per Caffetti *et al.* (2008) [16]. This record is linearly related to the frequency of DNA strand breaks in the cell population in a study on a wide range of damage. The primary damage score was calculated in arbitrary units (AU) as the sum multiplying the number of observed nucleoids in each class by the class value (0, 1, 2 or 3). The results were reported as the averages in AU with their corresponding standard error of the mean (SEM).

2.6. MN and NAs Test

Approximately 0.3 cm^3 of blood from each fish was smeared on a slide coded (blood extensions). Three smears were performed for each exposed individual. The material was fixed in absolute ethanol for 10 min and dried for

24 h. After wards, the material was hydrolysed in 1 N HCl for 11 min in moist chambers at 60˚C. After this, the slides were washed in distilled water and placed in Schiff's reagent for 2 h. A total of 3000 erythrocytes form the fish were analysed (blindly) under an immersion objective (1000X). For the identification of MN, some criteria were adopted according to Huber and Streng (1983) [17], and the NAs were recorded according to the classification of Carrasco *et al.* (1990) and Bolognesi and Hayashi (2011) [10] [11]. The frequency and SEM values for MN and other NAs in the cells were quantified.

2.7. Statistical Analysis

All data were expressed as the mean ± SEM. The data do not follow a normal distribution (Shapiro-Wilk) and Kruskal-Wallis test show differences between groups. The results obtained in the comet assay and the MN and NAs test were compared with the NC and all groups by the non-parametric Mann-Whitney test. The program used was the Statistical Package for the Social Sciences for Windows, version 15.0, (SPSS Inc., Chicago, IL, EUA).

3. Results

3.1. Comet Assay

The results obtained using the comet assay in erythrocytes of *O. niloticus* exposed to different IMI concentrations and their respective NC and PC is presented in **Table 1**. The primary DNA damage was expressed in AU (means and SEM) and represents strand breaks, alkali-labile sites, and delayed-repair sites as initial damage. The erythrocytes of the NC group show a higher occurrence of classes 0 and 1 presented lower genotoxic damage than those that underwent PC and IMI treatments. Fish injected with cyclophosphamide presented erythrocytes with DNA damage index statistically significant in relation to NC. The erythrocytes observed revealed a higher rate of DNA damage (expressed in AU) in all groups exposed to IMI compared to the NC and did not find statistically significant differences between the AU values obtained in the different IMI concentrations.

3.2. MN and NAs Test

The means and SEM of MN and other NAs in erythrocytes of *O. niloticus* exposed to IMI and their respective NC and PC are shown in **Table 2**. The erythrocytes of the NC group show a low frequency of MN and other NAs. Higher chromosomal damage level was found on the PC, with a frequency of MN statistically significant in relation to NC. Also, statistical differences in MN frequency and some NAs such as blebbed nuclei (BL) and notched nuclei (NT), were observed between NC and the highest IMI concentration.

4. Discussion

The impact of IMI use under field conditions has not been well studied. The evaluation of the action of different concentrations of IMI and its consequences on the genetic material of fish is of the utmost importance. The

Table 1. Mean ± SEM expressed in arbitrary units (AU) of the primary DNA damage in the fish *Oreochromis niloticus* exposed to three concentrations of IMI and cyclophosphamide.

Groups	Classes				
	0	1	2	3	AU
NC (H_2O)	75.1 ± 7.9	19.7 ± 4.5	5 ± 2.8	3.4 ± 2.0	39.9 ± 12.4
PC (20 mg/mL)	20.7 ± 8.0	64.6 ± 6.4	11.7 ± 3.8	5.9 ± 2.0	105.7 ± 13.8*
IMI (250 µg/L)	19.3 ± 4.5	54.8 ± 6.7	13.7 ± 2.5	13.6 ± 3.9	123 ± 16.1*
IMI (125 µg/L)	28 ± 7.4	58.6 ± 5.3	9.3 ± 2.2	7.5 ± 1.9	99.7 ± 13.7*
IMI (62.5 µg/L)	36.7 ± 11.6	35.6 ± 5.8	17.4 ± 4.8	12.1 ± 4.1	106.7 ± 11.8*

NC: negative control; PC: positive control (cyclophosphamide); IMI: imidacloprid; AU: arbitrary units; $^*p < 0.05$, values statistically significant, compared to negative control with the Mann Whitney test.

Table 2. Mean ± SEM of the frequencies of various erythrocyte abnormalities in 3000 erythrocytes from the fish *Oreochromis niloticus* exposed to three concentrations of imidacloprid (IMI) and cyclophosphamide.

Groups	Other nuclear abnormalities		
	MN	Blebbed Nuclei	Notched Nuclei
NC (H_2O)	1 ± 0.4	0.4 ± 0.3	0 ± 0
PC (20 mg/mL)	3.7 ± 0.8*	1.1 ± 0.3	0.5 ± 0.4
IMI (250 µg/L)	2.8 ± 0.8*	3.1 ± 1*	3.8 ± 0.8*
IMI (125 µg/L)	1.4 ± 0.7	2.4 ± 0.8	2.9 ± 1.6
IMI (62.5 µg/L)	0.8 ± 0.4	0.4 ± 0.3	0.7 ± 0.4

NC: negative control; PC: positive control (cyclophosphamide); IMI: imidacloprid; MN: frequency of micronuclei; *p < 0.05, values statistically significant, compared to negative control with the Mann Whitney test.

imidacloprid is one of the most widely used and sold neonicotinoids for the control of pests in different areas, however, it affects not only insect-pests but also non-target organisms. It can cause toxicity in ecosystem organisms, such as earthworms [18], amphipods, microalgae [19] and crustacean [20] among others. Also, the application of this insecticide has been associated with the death of bees in different regions of the country [21] [22]. Besides toxicity studies, it has been evaluated the genotoxicity in plants and animals. For example, DNA damage and increased frequency of MNs in frogs *Hypsiboas pulchellus tadpoles* (Anura, Hylidae) [23] and chromosomal aberrations and MNs in *Allium cepa* and *Tradescantia pallida* [12], showed genotoxic effects.

Different species of fish are used for evaluating pesticides and the choice of blood (erythrocyte) is made primarily because these cells are easily collected without sacrificing the animal and do not require additional separation methods [24] [25]. The use of different IMI concentrations caused a statistically significant increase ($p < 0.05$) in damage to the DNA molecule. This damage refers to strand breaks, alkali-labile sites, and delayed-repair sites, which indicates the genotoxic potential of the insecticide for this aquatic organism. The comet assay has been widely used in studies with pesticides; such a test was applied to the fish species *Channa punctatus* (Channidae) when exposed to atrazine. In this paper, the authors observed an increase in DNA damage in the erythrocytes of these fish [26]. The same results were observed in studies with the phorate pesticide, a genotoxic organophosphate, in fingerlings of *Labeo rohita* (Cyprinidae) [27], and Roundup® herbicide, evaluated in *Anguilla anguilla* (Anguillidae), also displayed genotoxicity [28]. The results obtained in this study corroborate previous studies with other pesticides and support the claim that the comet assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants.

With the increased use of pesticides against harmful plants and insects in the last decade, it has been observed that certain agricultural chemicals can cause changes that include the inhibition of cell division, the induction of chromosomal abnormalities and chromosomal damage [29]. Chromosomal aberrations induced by pesticides from different cultures are widely used as an indicator of genetic damage. The MN test associated with NAs is one of the most promising tests in ecotoxicological evaluations and the best one for the observation of damage at the chromosomal level [11]. This assay has been widely applied in measurement studies of genotoxicity of different chemicals in species of fish and the biological monitoring of contaminated areas [30] [31]. Based on the results obtained in this study, IMI caused a dose-dependent increase in the frequency of MN and other NAs as BL and NT, which was statistically significant ($p < 0.05$) in the highest concentration evaluated (250 µg/L) compared to the NC. The results obtained in this study corroborate other pesticide studies using different species of fish, such as the evaluation of Aficida® and Endosulfan insecticides in fish erythrocytes from *Cnesterodon decemmaculatus* (Poeciliidae) and *Carassius carassius* (Cyprinidae) by the MN test [32] [33]. These studies demonstrate the effectiveness of fish and the MN test as a model for the biomonitoring of aquatic ecosystems that may be affected by pesticides.

Different kinds of NAs are frequently observed in fish erythrocytes, although the mechanisms responsible have not been fully explained. The BL often is considered to be indicators of genotoxic damage and other NAs, such as NT nuclei, are mainly associated to cytotoxicity [11].

Several authors confirm that the comet assay is slightly more sensitive than the MN test in detecting early genetic damage. Furthermore, the comet assay identifies repairable DNA lesions; consequently, only a limited por-

tion of the induced primary DNA damage is assumed to lead to the serious damage represented by MN. The MN test detects unrepaired chromosome breaks while the comet assay detects strand breaks, alkali-liable sites, and delayed-repair sites that may or may not become repaired. A combination of MN test and comet assay enables comparison of the relative sensitivity of the two test systems [34]. A study in tadpole shows that IMI concentrations increased the frequency of primary DNA lesions estimated by comet assay. Additionally, the data revealed that the comet assay was more sensitive than the MN test in detecting early DNA damage when the same IMI concentrations were employed for tadpole exposure [35].

Pesticides can induce oxidative stress by generation of free radicals that interact with cellular membrane cause lipid peroxidation, alternations in membrane fluidity, DNA damage and finally carcinogenic effects [36]. For example, study indicates potential of IMI to develop oxidative stress and DNA damage in silkworms [37]. The significant increase in the lipid peroxidation can be possibly due to the reactive oxygen intermediates (ROS), which may lead to cell apoptosis [38]. Previous investigations have reported the induction lipid peroxidation by other pesticides such as endosulfan [39] and cypermethrin in fish [40].

The IMI has the potential to reach surface waters; it has been estimated to potentially reach such waters in concentrations up to 36 µg/L and has been detected in surface waters at concentrations up to 14 µg/L [41]. Higher concentrations can reach the water after application of this insecticide in crops of sugarcane and cause genotoxic damage to non-target organisms such as fish.

5. Conclusion

The results demonstrated that the concentrations tested in the comet assay induced primary damage to the DNA by increasing the frequency of strand breaks and alkali labile sites and increasing the frequency of MN at the highest concentration tested. Imidacloprid induced primary DNA damage at the concentrations tested and damage at the chromosomal level in the concentration of 250 µg/L. The results of this study help illustrate the potential ecological risk of IMI in aquatic environments and indirectly, to human health. The development of strategies for reduction in pesticide application and decreasing its impact on fish and other aquatic animals is necessary. This is a laboratory study previous to other field studies to be performed in areas where is applied this pesticide. While it is impossible to prevent the use of pesticides by humans, it would be beneficial to decrease the doses applied to farmland or to encourage the development of less toxic substances with similar effects that will enable future generations to live in healthier environments.

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ANEXO 4

Liver alterations in *Oreochromis niloticus* (Pisces) induced by insecticide imidacloprid: histopathology and heat shock protein *in situ* localisation

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Abstract

The liver is very sensitive to environmental contaminants such as pesticides, being the first targets of toxicity of a substance. The objective of this study was to investigate the possible effects of imidacloprid (IMI) concentrations an insecticide used for growing sugarcane in liver of *Oreochromis niloticus*. A semi-quantitative analysis for histopathological alterations of IMI on the liver was performed by light microscopy and cellular labelling of heat shock proteins (HSP70) by immunohistochemistry. The most common liver changes at all concentrations of IMI were hydropic degeneration, pyknotic nuclei and loss of cell limits. Steatosis and increased levels of HSP70 were detected in the hepatocytes with the highest concentration of IMI. In conclusion, the tested concentrations of IMI induced histopathological changes in the liver of *O. niloticus* and active defence mechanisms to maintain the morphophysiological integrity of liver. This insecticide has a toxicity potential for these fish, which is a non-target organism for its action.

Keywords: pesticides, tilapia, histochemistry, HSP70.

Introduction

The production of sugarcane has expanded in the last decade and is used for biofuels, such as ethanol, which is one of the most employed fuels today. [1] Brazil is now the largest producer of sugarcane in the world and approximately 50% is destined to be used for ethanol. [2] The harvest of 2013/2014 was 653.519 thousand tons of sugar and 27.543 thousand m³ of ethanol. [3] The increase in sugarcane crops has been

accompanied by an increase in the consumption of pesticides, and their indiscriminate application is one of the greatest current concerns. Among these, the neonicotinoid insecticides are among the most widely used, with record use in more than 120 countries. [4] The pesticide with the greatest success is imidacloprid (IMI), the best-selling insecticide for many years and the second most widely used pesticide in the world. [5] In 2010 alone, the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA) reported sales of 1.934 tonnes of IMI. [6] This was used to control pests such as sucking insects of the soil, chewers and termites, and they were used in 140 different cultures such as soybeans, sugarcane, citrus, cotton, coffee, tomatoes, and lettuce. [7]

In soil, the IMI is biotransformed into several metabolites and degrades completely into carbon dioxide. [8] Its presence in the aquatic environment is increasingly worrying because of the excessive use of commercial products formulated with this substance. [9] Imidacloprid can accumulate and generate high risk to the environment and reach non-target organisms. [10]

Fish are widely used as effective models of water pollution because they accumulate contaminants and show a biochemical, cellular, physiological and histologically differentiated response, making it an excellent biomarker with high application for environmental monitoring. [11, 12] Among the most commonly used species of fish is *Oreochromis niloticus*, known as the Nile tilapia, the second most cultivated species of fish in the world. [13] This has been considered optimal in laboratory studies for its many advantages such as a high rate of growth and reproduction, resistance and tolerance in laboratory conditions and adaptation in commercial food. [14, 15]

The effects of contaminants in fish can manifest in organs and tissues. Histological changes could be used as sensitive tools that can be detect the direct toxic effects of

various compounds, and they act as good markers of environmental stresses. [16, 17] Biomarkers in cellular and molecular level complement histopathology study, offering more information on possible mechanisms of action of pesticides on non-target organisms. Heat shock proteins (HSP) are activated as a primary protection response the organisms against any change induced environmental stress. [18]

Thus, the aim of this study was to evaluate the toxic potential of IMI in *O. niloticus* livers through histological, histochemical and immunohistochemistry approaches.

MATERIAL AND METHODS

Chemical

Chemical compound IMI (1-[(6-chloro-3-pyridinylmethyl]-N-nitro-2-imidazolidinimine; CAS: $N^0$138261-41-3; molecular formula $C_9H1_0C_lN_5O_2$) was obtained from Bayer Crop Science (Agricultural Experiment Station-SP, Lot EDE 0036241).

Test organism

The test organism used in the experiment was the Nile tilapia, *O. niloticus* (Perciformes, Cichlidae). Individuals (n=40) that were two months of age, weighing 30±2g and12-15 cm in size were used to avoid intraspecific differences associated with size and age. The individuals analysed were reared on fish culture farms and kept in the Experimental Garden of the Institute of Biosciences, UNESP (São Paulo State University), Rio Claro, São Paulo, Brazil. These animals were acclimated in tanks under controlled conditions,

with filtration, aeration systems at an average temperature of 23ºC and fed with commercial food.

Experimental design

The bioassays were set up in aquariums (40L) (size: length= 45cm, height= 25cm, width= 20cm). The fish were exposed for 96-h, according to other studies with fish [19-21] and with constant aeration at a temperature of 23 ± 2°C, pH=8.3 and photoperiod of 14-h light/10-h darkness. Eight aquariums were used in the experiment; i.e., two for controls groups (fresh water) and the remaining six were exposed to the three IMI concentrations (C1: 250; C2: 125 and C3: 62.5µg/L). All treatments consisted of two replicates with five organisms each. The test concentrations were determined by the concentration used for growing sugarcane, based on previous studies. [22]

Dissection of animals

The study was approved by "The Ethics Committee on Animal Use", UNESP, and filed with the number 8937. After 96-h of treatment, fish were removed from aquaria to proceed to the dissection. They were anesthetized, euthanized by pithing with surgical scissors, and dissected in saline solution for liver extraction. Liver samples were cleared from adhering tissues, fixed with: aqueous Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid) (for histology, total protein and polysaccharides determinations), formal calcium (40% formalin, 10% $CaCl_2$ and distilled water) (for lipids determinations) and buffered paraformaldehyde (4% paraformaldehyde in 7.4

PBS) (for immunolabelling of HSP70). The material remained in the fixative for over 24 hours.

Histology and histochemistry

Portions of liver were dehydrated through ascending grades of alcohol (70%, 80%, 90% and 95%) for 15 minutes. The tissues were embedded in resin (Leica Historesin-Embedding Kit, according to the manufacturer's specifications) for 48-h in the refrigerator and transferred to plastic moulds with resin. The resin blocks with the material were cut serially using a Leica microtome (6µm thick sections). The sections were floated in a tissue floatation bath at 45°C and placed on glass slides. The sections were stained with haematoxylin and eosin (HE) according to histological procedures following the protocol of Junqueira and Junqueira. [23] For the histochemical analysis, the material was subjected to bromophenol blue for the detection of proteins. [24] Additionally, Periodic Acid-Schiff (PAS) and PAS simultaneously with Alcian blue techniques detected the presence of neutral and acid polysaccharides and Sudan black B and Nile blue was used for lipids, all according to the protocol of Junqueira and Junqueira. [23] Two slides with eight sections of each fish were analysed under a light microscope and photographed.

Semi-quantitative analysis of histological results

A total of 80 slides and 640 non-consecutive histological sections were analysed. The description and evaluation of the histological alterations were analysed in accordance with the Bernet protocol [25] with modifications. [26] Two parameters were established to

determine the index of alterations; i.e., the scores (α) and importance factors (w). Histopathological observations were classified in scores on a scale of 0–6 depending on the degree and extension of the alteration: (0) no occurrence, (2) mild occurrence, (4) moderate occurrence and (6) severe occurrence. The control group and each treatment performed were given a score for each alteration and for each individual. The importance factor was set for each lesion according to their pathologic importance (how it affected organ function and fish survivability). The alterations were classified as one of three important factors: (1) minimal pathological importance (damage is easily reversed); (2) moderate pathological importance (damage is reversible in most cases); and (3) severe pathological importance (damage is usually irreversible, leading to partial or total loss of the organ function). Multiplication of the score by the importance factor of each alteration was calculated to establish the index of alterations (I) for each individual using the equation: $Index_{ind} = \Sigma (W \times \alpha)$. From the individual indices, the mean and standard deviations (SD) were calculated for all groups. The results were compared between the control group and the groups with the three concentrations using the non-parametric Mann-Whitney test and the Statistical Package for the Social Sciences for Windows, version 15.0, (SPSS Inc., Chicago, IL, EUA).

Histological preparation for HSP70 determination

Liver samples were fixed in buffered 4% paraformaldehyde solution for 24-h, then slowly dehydrated in ascending series of ice buffered alcohol (pH 7.4) (15, 30, 50, 70, 85, 90, 95, 100%, xylene (I) (II) and xylene) for 20 minutes and embedded in paraplast blocks. Sections of 6µm were cut serially using a microtome and floated in a tissue floatation bath at 45°C and placed on glass slides for immunohistochemistry.

Immunohistochemistry for the detection of HSP70

The description and evaluation were analysed in accordance with the procedure used for Silva-Zacarin [27] with modifications. Paraffin wax was removed from slides with xylene and absolute alcohol. Dewaxed sections were then permeabilized with Triton X-100 to ensure free access of the antibody to its antigen. These sections were washed in PBS (phosphate buffer saline) and incubate with primary antibody against HSP70 (anti heat shock protein 70, monoclonal antibody produced in mouse obtained from Sigma). For the control sections the primary antibody was omitted. After incubation overnight at 4ºC, sections were washed in PBS and covered with a secondary antibody (Anti mouse IgG whole molecule, obtained from Sigma) conjugated to alkaline phosphatise and incubated for 1 hour at room temperature in a humidified chamber. The EnVision System alkaline phosphate kit (Dako™) was used in accordance with the instructions to obtain a red permanent coloured precipitate that indicate the enzyme-substrate reaction product. Quantitative analyses were carried out of immunostaining of HSP70 stress protein in the liver of the fish through the program Image J. For each individual, the averages and standard deviations were calculated for all groups. Analysis statistical was performed by Test-T, $p < 0.05$, according with obtained in the normality test of Shapiro-Wilk.

RESULTS

Histological analysis

During the experiments, no fish died and no symptoms of toxicity were observed. The fish of control group showed typical features of liver tissue (Fig. 1.1). As described for the species, the liver has exocrine pancreatic acini and hepatocytes of polyhedral shape arranged in rows with well-defined nuclei, some with homogeneously stained cytoplasm and other less homogeneous, interspersed with sinusoidal, in which erythrocytes are observed. In the groups treated with IMI, different alterations were observed in the hepatocytes. Table 1 lists the alterations observed with the corresponding importance factors (w). The most frequent and statistically significant alterations ($p < 0.05$) compared to the control group (Table 2) were hydropic degenerations, pyknotic nuclei (Fig. 1.2) and loss of cell limits (Fig. 1.3). In some fish (six), vacuolization of cytoplasms were observed in hepatocytes (Fig. 1.4), but were not statistically significant.

Histochemical analysis

The vacuolization of the cytoplasm observed by HE was analysed for specific histochemical staining. The tests used for histochemical detection of proteins and polysaccharides showed no differences between the treated and control group. In the analysis of lipids by Nile blue staining, a difference between the controls (Fig. 2.1) and the highest concentration of IMI employed was observed. An increased presence of neutral lipids was observed (reddish colour) and acids (blue) in the cytoplasm of hepatocyte fish exposed to the highest concentration (Fig. 2.2).

Detection of HSP70 by immunohistochemistry

Results of detection of HSP70 are shown in (Fig. 3). It was found that the control group had immunostaining of HSP70 protein, indicating that the protein is constitutively expressed in this organ as a molecular chaperone. In the treated groups, HSP70 immunolabelling was higher and statistically significant in the highest concentration of IMI.

DISCUSSION

Some time ago, environmentalists and government officials increased actions for water resource protection worldwide. Several decisions were based on the scientific literature whose main objective was to assess the action of several potentially toxic products on the water bodies. In addition to contamination of the water, the poisoning of aquatic animals is a concern. In this context, the presence of products that are not designed specifically for use in bodies of water is highly disturbing. However, they enter the aquatic environment through spraying practices, draining or leaching, and they reach non-target organisms, such as fish.

The neonicotinoid insecticides have a potential for leaching and several studies have characterized their presence in water. [28] A wide variety of these insecticides have been found in water bodies such as tanks, lakes, groundwater and streams. [6, 29] The concentrations detected in water vary depending on the study; for example, surface waters in the United States of America showed that 50% of the collected water samples had a presence of IMI and neonicotinoids low biodegradation in water. [30] Another study in California showed that 89% of the water samples contained IMI and 19% exceeded the reference index established by the US Environmental Protection Agency

(USEPA) Aquatic Life Benchmark. [29] In Sydney, Australia a concentration of 4.56 μg/L of IMI in rivers was reported. [31]

For these reasons, studies conducted on aquatic organisms are of great importance to predict the possible environmental effects of IMI. Fish are organisms widely employed in studies to assess the pesticide formulations. [32-34] Pathological changes in fish are the result of biochemical and physiological changes unfavourable to the body. [35, 36] Liver histology is a useful tool for evaluation of the relationship between damage in fish and environmental pollution. [27, 37-39] The liver is a key organ for the determination of pollutant actions as it is very sensitive to environmental contaminants, as well as detoxification of endogenous and exogenous products such as pesticides. The hepatocytes are considered prime targets for toxicity of a substance. [40] Multiple histological studies have been carried out on fish exposed to different insecticides. For example, studies in *Rhamdiaquelen* exposed to Folidol 600® (active ingredient is methyl parathion) showed there were several cellular changes in the liver, simulating what happens in the natural environment when water bodies are contaminated with this product. [41] Banaee et al. [42] performed chronic treatment of diazinon in *Oncorhynchus mykiss*, which caused morphological alterations in the liver. Another example is the acute and chronic exposure to endosulfan, which caused a significant impact on *Channa punctatus* liver tissue, showing that the fishes are very sensitive to the presence of this insecticide. [43]

Various histological alterations were observed in the groups exposed to IMI. Hydropic degeneration, pyknoticnuclei and loss of cell limits showed statistically significant differences when compared with the control group. The hydropic degeneration is characterized by an increase in cell volume due to the accumulation of water and electrolytes inside the cell. [44] The pyknotic nucleus with reduced diameter is a change

that indicates condensation of chromatin, suggesting onset of the cell death process. The loss of cell limits can result in drastic changes in organ function. [45]

The alterations observed here have been detected in other studies with other insecticides, such as fenvalerate in *Cirrhinus mrigala* [46], endosulfan in *Cichlaso madimerus* [47], deltamethrin in *Oreochromis Niloticus* [48] and cypermethrin in *Oreochromis mossambicus.* [49] Effects of the mix of benzo (a) pyrene (BaP), dichlorodiphenyltrichloroethane (DDT) and tributyltin (TBT) were evaluated in *Rhamdia quelen* [38] , suggesting that the presence of these compounds may affect metabolic processes and produce pathologic lesions in the liver, which is the central organ of detoxification.

Many of these studies related liver damage to increased biochemical biomarkers related to oxidative stress. [38, 50, 51] Free radicals are important components in the toxic effects of pesticides and other environmental chemicals that induce oxidative stress by interacting with biomolecules. [52]

Additionally, some cells were found with corresponding lipid storage vacuolization (Fig. 2.2) and signs of degradation. This is a steatosis (accumulation of lipid droplets).

This may be a failure of lipid metabolism due to exposure to IMI. The liver, muscle and adipose tissue are the main organs for the deposition of lipids, and their accumulation is the result of the balance between lipogenesis and β oxidation, with many enzymes involved in these metabolic processes. [53] Increased synthesis and uptake of fatty acids brings with it increased accumulation of lipids in this organ. [54, 55] In most species of fish, lipids are predominant sources for obtaining energy, but excessive deposition of lipids can cause problems for the animal's health. [56] Several studies have indicated that exposure of fish to environmental stress can disrupt lipid metabolism, and they observed vacuole accumulation in the liver. [53-57]

The determination of biomarker at level molecular, complement histopathology study. The first response of an organism to any change-induced environmental stress happens at sub-cellular level and it could be detected by means of immunohistochemistry methods. These responses activate the antioxidant defence systems and heat shock proteins which are a primary protective response. [18] The HSP70 proteins play an integral role in the cellular response pathways to stress in most organisms, including fish. [58, 59] Alterations in fish hsp70 transcription levels in liver may serve as a rapid, reproducible, sensitive and simple ecotoxicological biomarker. [60] In liver hsp70 genes expressions are related with the development of antioxidant and detoxification responses. [61] The highest concentration of HSP70 in liver occurs in hepatocyte cytosol, but it can be translocated to nucleus and mitochondrial membrane in response to a toxic exposure. [62] In fish, hsp70 can be induced by exposure to pesticides. [63] Oxidative stress can induce hsp70 mRNA expression, indicating that oxygen radicals contribute to the expression of HSP70. [64, 65] Probably the biggest concentration of IMI, activates the expression of hsp70 in response to oxidative stress generated by this pesticide strengthening tolerance to environmental stress. [66]

Many studies have indicated that environmental stressors, such as pesticides, modulate hsp70 expression in fish. [63, 67, 68] Some authors relate the induction of the expression of this protein with stress conditions caused by pesticides. [69, 70]

In this study, increased *in situ* localization of HSP70 in liver, mainly in the highest concentration of IMI, may be related to proteotoxicity in hepatocytes presumably induced by oxidative stress the occurred by insecticide exposure, which shows correlation with histopathological results observed in liver. It can be concluded that the stress-inducible HSP70 could emerge by inducing of hsp70 gene expression and, consequently, the increase of immunolabelling of HSP70 in tissue in order to refolding

damage proteins as a protective mechanism in response to liver microscopic lesions induce by IMI.

The Convention on Biological Diversity (2002) showed a loss of diversity as one reason for the loss and degradation of habitats rich in species in many developing countries. The increased use of neonicotinoids may play a role in the decline of populations. The accumulated concentrations in soil, water, plants, nectar and pollen are enough to subject large groups of organisms to environmental risk. We should evaluate possible strategies for achieving a balance between the use of neonicotinoids to meet the demands for the production of food and fuel and the need for managing the world's biodiversity to ensure the health of ecosystems.

CONCLUSIONS

The tested concentrations of IMI induced histopathological changes in the liver of *O. niloticus* and the semi-quantitative analysis used in this study proved to be an important tool for assessment of adverse effects in liver induced by toxic processes. In addition the highest concentration of IMI produces steatosis and activated cytoprotective mechanisms mediated by heat shock protein 70 to maintain the morphophysiological integrity of the liver. Determination of Hsp70 levels in field of ecotoxicology can be used as a biomarker of effect in fish liver exposed to IMI insecticide that could be applied in environmental risk assessment of pesticides for aquatic animals (i.e. fishes).

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FIGURE CAPTIONS

Figure 1: Liver sections of *O. niloticus* stained with hematoxylin-eosin. Control Group (1) and treatment groups (2, 3, 4). Bars: 20µm; pa: pancreatic acini; h: hepatocytes; n: nucleus; e: erythrocytes; hd: hydropic degeneration; pn: pyknotic nucleus; asterisk: loss of cell limit; vc: vacuolated cytoplasm.

Figure 2: Liver sections of *O. niloticus* stained with Nile blue. Bars: 20µm. Control group (1) and IMI treatment group (2) shows vacuoles reddish colour.

Figure 3: Relative level of HSP70 (mean±SD) in *O. niloticus* (Pisces) livers. C: Control group (fresh water); IMI [C1]: imidacloprid (250µg/L); IMI [C2]: imidacloprid (125 µg/L); IMI [C3]: imidacloprid (62.5µg/L). *$p < 0.05$, significant differences compared with the control group, as shown by the Test-T.

Figure 1

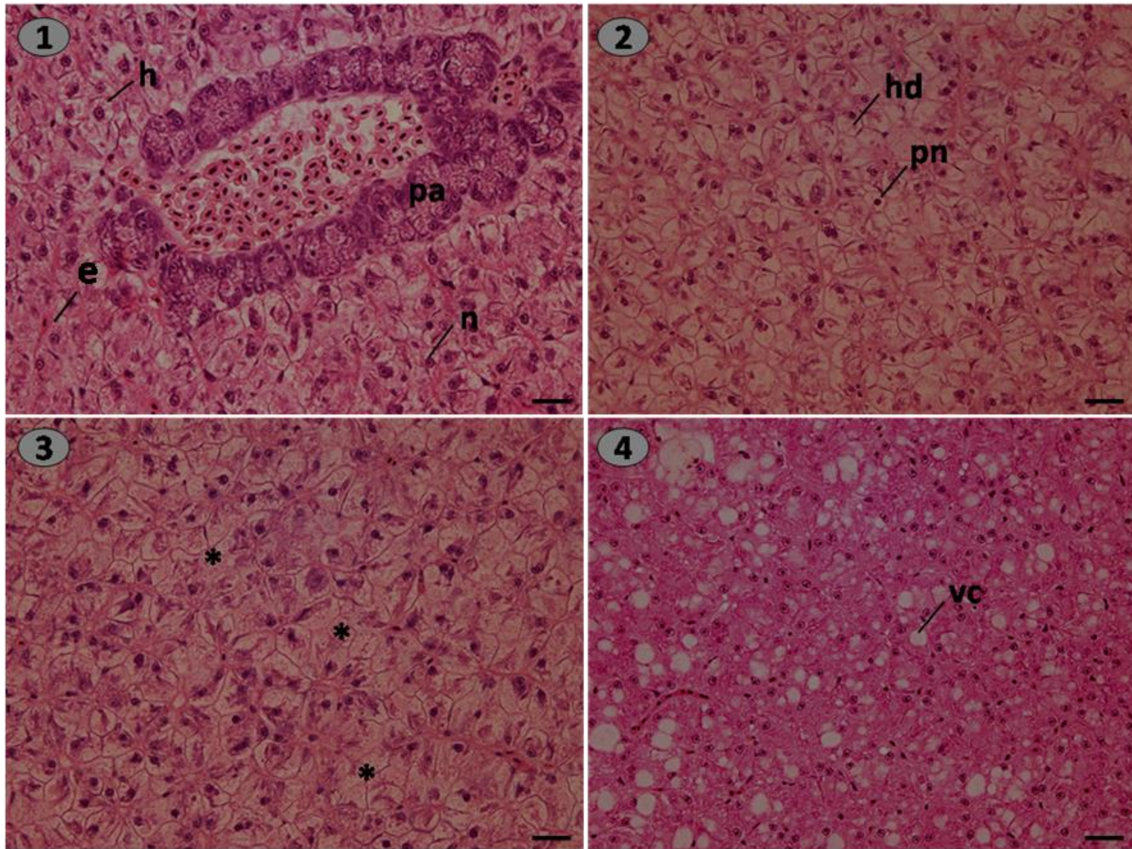

Figure 2

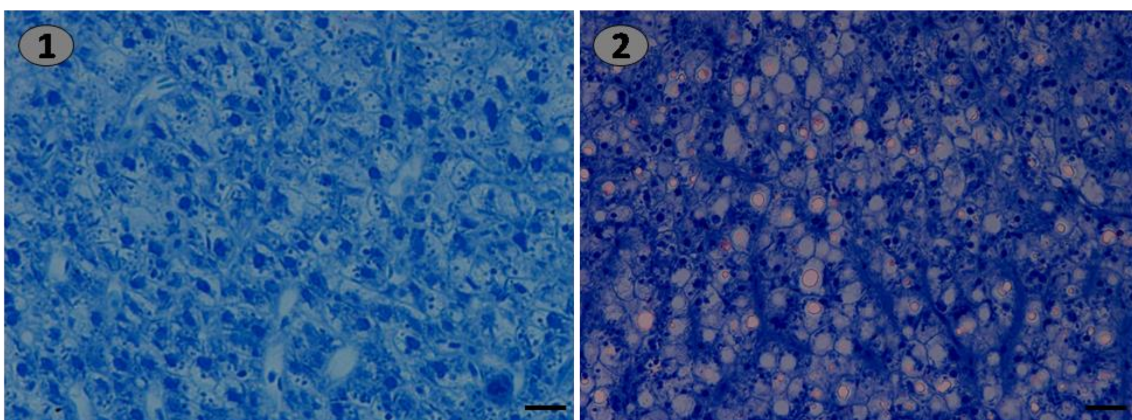

Figure 3

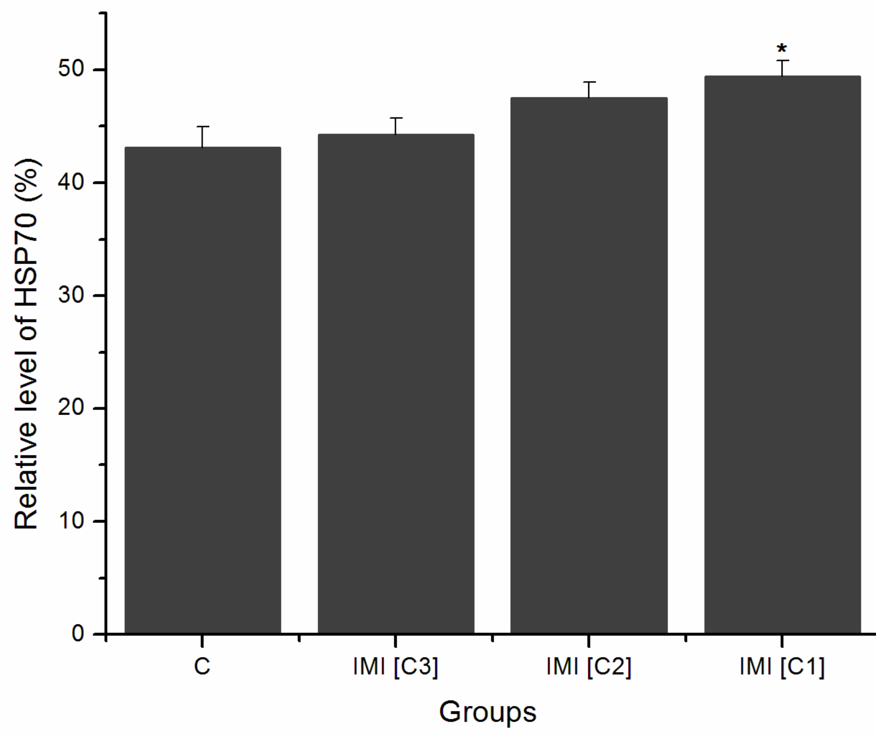

Table 1: Alterations observed in *O. niloticus* livers exposed to IMI and their corresponding factor of importance.

	Characteristics examined	Factor of importance (w)
Hepatocytes	Hydropic degeneration	2
	Pyknotic nucleus	3
	Loss of cell limit	3
	Vacuolated cytoplasm	1
	Nuclear alterations	2

Table 2: Frequency of occurrence of significant liver alterations found in *O. niloticus* exposed to IMI.

Liver alterations	Control group	IMI [C1]	IMI [C2]	IMI [C3]
Hydropic degeneration	1.6± 0.6	8.0±1.0*	8.4±0.9*	6.0±0.9*
Pyknotic nucleus	0±0	6.6±0.6*	8.4±1.0*	5.4±0.6*
Loss of cell limit	0.6±0.6	9.6±1.3*	11.4±1.4*	9.0±1.0*
Vacuolated cytoplasm	1.8±0.7	2.2 ± 0.7	2.4±0.7	1.6±0.5
Nuclear alterations	0.4±0.4	1.6±0.6	3.2±0.5	1.6±0.6

Results presented as mean±SD. IMI [C1]: imidacloprid (250 μg/L); IMI [C2]: imidacloprid (125 μg/L); IMI [C3]: imidacloprid (62.5 μg/L). * $p < 0.05$. Values statistically significant, compared to control group with the Mann-Whitney test.