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Darlene Denise Dantzger

Estudy of the toxic effects of insecticide diflubenzuron its metabolite p-chloroaniline and their mixtures in tilapia and zebrafish, in the presence and absence of soil

Estudo dos efeitos tóxicos do inseticida diflubenzuron seu metabólito p-cloroanilina e suas misturas em tilápia e zebrafish, na presença e ausência de solo

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COMISSÃO EXAMINADORA

Prof. Dr. Hiroshi Aoyama

Prof.(a). Dr.(a). Marta Siviero Guilherme Pires

Dr.(a). Thaís Franchini Tornatore

Dr.(a). Vera Lucia Scherholz Salgado de Castro

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RESUMO

O inseticida Diflubenzuron (DFB), utilizado por muitos piscicultores pela sua eficácia contra parasitas de peixes, quando metabolizado ou degradado produz a p-cloroanilina (PCA) um composto extremamente tóxico. Uma vez no ambiente aquático, esses compostos podem formar misturas e sua biodisponibilidade depende de fatores como a presença de solo. Diante desta problemática o objetivo geral desta tese foi avaliar os efeitos tóxicos dos compostos DFB e PCA, individualmente e, em diferentes proporções de suas misturas, na presença e ausência de solo, nos peixes *Oreochromis niloticus* (tilápia) e *Danio rerio* (zebrafish). Nos ensaios com as tilápias, inicialmente foram obtidas as concentrações dos compostos isolados e de suas misturas que foram letais para 50% dos peixes (CL50-96h) na presença e ausência de solo. As misturas do DFB com PCA foram testadas nas proporções contendo 75%, 50% e 25% de PCA. Com estes resultados, foram realizados os cálculos de índice de aditividade que nos forneceram o tipo de interação que ocorreu entre cada mistura dos compostos. Os mesmos valores de CL50-96h serviram de base para o cálculo das concentrações subletais dos compostos isolados e suas misturas, sendo as tilápias expostas por 96h para a avaliação enzimática. As atividades das enzimas fosfatases ácida (AcP), alcalina (AlP) e catalase (CAT) foram avaliadas nas brânquias destes peixes e as mesmas enzimas, incluindo a alanina (ALT) e aspartato (AST) aminotransferases, foram dosadas do fígado destes organismos. Resumidamente, o solo diminuiu a biodisponibilidade dos compostos em água e ocorreu uma relação antagonística para a mistura com 25% PCA e sinérgica para a mistura com 75% deste composto. Os peixes apresentaram estresse oxidativo e possíveis danos nas brânquias e fígados com alterações na atividade das enzimas CAT, AcP, AlP, AST e ALT. Para os testes com o zebrafish, inicialmente os embriões com 1 hora pós-fertilização (hpf), larvas (96 hpf) e adultos foram expostos a 0, 2.2, 4.4, 9.7, 21.3, 46.8 e 100 mg/L dos compostos isolados e suas misturas durante 96 horas. Após as 96h os organismos mortos foram anotados para determinar a CL50-96h e alterações no desenvolvimento do embrião até a fase larval foram observadas. Dentre as misturas avaliadas a com 75% de PCA causou mais mortalidade dos organismos, seguida das misturas com 50% e 25% de PCA. Os embriões com 1 hora pós-fertilização (hpf) expostos a misturas de DFB e PCA por 96 h apresentaram atraso na eclosão e anormalidades como edema pericárdico, edema de saco vitelino e malformação da coluna vertebral. As larvas com 144 hpf expostas ao PCA e às misturas com 50% e 75% de PCA apresentaram alterações na morfologia do fígado, no intestino e na bexiga natatória. A presença de DFB diminuiu os efeitos tóxicos da PCA para os organismos e a mistura contendo 25% de PCA mostrou efeitos antagônicos. Os compostos

estudados mostraram efeitos deletérios sobre os organismos teste e, portanto, podem causar danos ao ambiente aquático e, consequentemente, aos seres humanos. Os resultados desta pesquisa poderão contribuir para uma melhor compreensão da toxicidade dos compostos e suas misturas, no ambiente aquático.

Palavras chaves: Diflubenzuron, p-cloroanilina, fosfatases, catalase, transaminases, mistura, solo, tilápia, zebrafish, embriões.

ABSTRACT

The insecticide Diflubenzuron (DFB), used by many fish farmers due to its effectiveness against fish parasites, when metabolized or degraded produces p-chloroaniline (PCA) an extremely toxic compound. Once in the aquatic environment, these compounds can form mixtures and their bioavailability depends on factors such as the presence of soil. The objective of this work was to evaluate the toxic effects of the compounds DFB and PCA, individually and, in different proportions of their mixtures, in the presence and absence of soil, on fish *Oreochromis niloticus* (tilapia) and *Danio rerio* (zebrafish). For the assays with tilapia, the concentrations of the isolated compounds and their mixtures that were lethal to 50% of the fish (LC50-96h) were obtained in the presence and absence of soil. The mixtures of DFB with PCA were tested in proportions containing 75%, 50% and 25% PCA. With these results, the additivity index calculations were performed to provide the type of interaction that occurred between each mixture of the compounds. The LC50-96h values were used for calculating the sublethal concentrations of the isolated compounds and their mixtures in which tilapia were exposed for 96 hours for the enzymatic evaluation. The activities of the acid (AcP), alkaline phosphatase (AlP) and catalase (CAT) enzymes were evaluated in the gills of these fish and these same enzymes, including alanine (ALT) and aspartate (AST) aminotransferases, were dosed in the liver of the animals. Summarizing the results, the soil decreased the bioavailability of the compounds in water and an antagonistic relationship occurred for the mixture with 25% PCA and synergistic for mixture with 25% of this compound. The fish presented oxidative stress and possible damages in the gills and livers with alterations in the activity of CAT, AcP, AlP, AST and ALT enzymes. For zebrafish assays, embryos with 1-hour post-fertilization (hpf), larvae (96 hpf) and adults were exposed to 0, 2.2, 4.4, 9.7, 21.3, 46.8 and 100 mg/L of the isolated compounds and mixtures thereof for 96 hours. After 96h the number of dead organisms was noted to determine the LC50-96h and alterations were observed in embryonary development and larval stage. Among the mixtures evaluated 75% PCA caused more mortality of larvae and adults zebrafish, followed 50% and 25% PCA. The embryos with 1hour post fertilization exposed to mixtures of DFB and PCA for 96 h presented a delay in hatching and abnormalities such as pericardial edema, yolk sac edema, and, spine malformation. The larvae (144 hpf) exposed to PCA and of the mixtures with 50% and 75% PCA have changes in liver morphology, gut, and swim bladder. The presence of DFB decreased the toxic effects of PCA to the organisms and the mixture containing 25% PCA showed antagonistic effects. The compounds studied have shown deleterious effects on the test organisms and therefore can

cause damage to the aquatic environment and, consequently, to humans. The results of this research can contribute to a better understanding of the toxicity of the compounds DFB, PCA and their mixtures, in the aquatic environment.

Keywords: Diflubenzuron, p-chloroaniline, phosphatases, catalase, transaminases, mixture, soil, tilapia, zebrafish, embryos.

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Sumário

INTRODUÇÃO GERAL

O diflubenzuron (DFB) (Figura 1) é um inseticida derivado da classe química benzoilfeniluréia que atua inibindo a síntese de quitina, polissacarídeo que constitui o exoesqueleto dos artrópodes e crustáceos, provocando morte do indivíduo por consequência da má formação da cutícula (Zaidi et al., 2013). Este composto difere amplamente dos inseticidas convencionais por provocar mudanças morfofisiológicas durante o processo de desenvolvimento e metamorfose do inseto, além de induzir efeitos morfogenéticos que podem resultar em completa inibição da emergência de insetos adultos (Macken et al., 2015).

O diflubenzuron foi registrado pela primeira vez como pesticida para controlar pragas em culturas de citrus, algodão e cogumelos nos Estados Unidos em 1976 (EPA, 1997). Desde então, vem sendo utilizado por diversos países na agricultura, na pecuária para o controle da mosca de chifres em gados e em programas de combate a mosquitos vetores de doenças como a dengue (Eisler, 1992; Macken et al., 2015; Pereira Maduenho and Martinez, 2008; Zaidi et al., 2013).

Os inibidores da síntese de quitina como o DFB não agem apenas em insetos considerados como pragas, assim, insetos benéficos e artrópodes como as aranhas, caranguejos, lagostas, camarões, dafinídeos e qualquer organismo que produza quitina será adversamente afetado pelo seu uso (Olsvik et al., 2013). Muitos organismos que são base da cadeia alimentar como os invertebrados de água doce, crustáceos marinhos e estuarinos, principalmente aqueles que apresentam exoesqueleto quitinoso, podem ser dizimados quando expostos ao DFB (Medeiros et al., 2013).

Devido a esta inespecificidade, o DFB mostrou eficácia em controlar crustáceos parasitas de peixes como *Lernaea cyprinacea* e *Dolops carvalhoi* e vem sendo indiscriminadamente utilizado por piscicultores de diversos países para este fim (Chang et al., 2017; Macken et al., 2015; Medeiros et al., 2013; Tomáš Scholz, 1999; Zaidi et al., 2013). A falta de produtos específicos para o controle destes crustáceos parasitas na piscicultura e a aparente baixa toxicidade aos peixes, tornou o uso do DFB cada vez mais frequente (Pereira Maduenho and Martinez, 2008; Tomáš Scholz, 1999). Apesar de não ser letal para diversas espécies de peixes, alguns estudos já demonstraram que este inseticida provocou alterações fisiológicas e bioquímicas que afetaram a saúde destes organismos (Benze et al., 2014; Olsvik et al., 2013; Pereira Maduenho and Martinez, 2008; Zaidi et al., 2013).

Em ambientes de água doce, a meia-vida do DFB pode variar de 8 a 29 dias dependendo da temperatura da água, do pH e da quantidade de matéria orgânica, sendo mais

estável a baixas temperaturas, pH alto e grande quantidades de matéria orgânica (Benze et al., 2014) . Alguns estudos mostraram que o DFB foi persistente em lagoas de criação de peixes apresentando meia-vida que variou de 115 a 170 dias (Roth et al., 1993; Samuelsen, 2016). Além disso, o diflubenzuron no meio ambiente pode sofrer metabolização ou degradação e gerar diversos compostos químicos, dentre estes a p-cloroanilina (PCA) (Figura 1), um composto considerado altamente tóxico para diversos organismos, incluindo os peixes e os seres humanos (Chang et al., 2017; Guoguang et al., 2001).

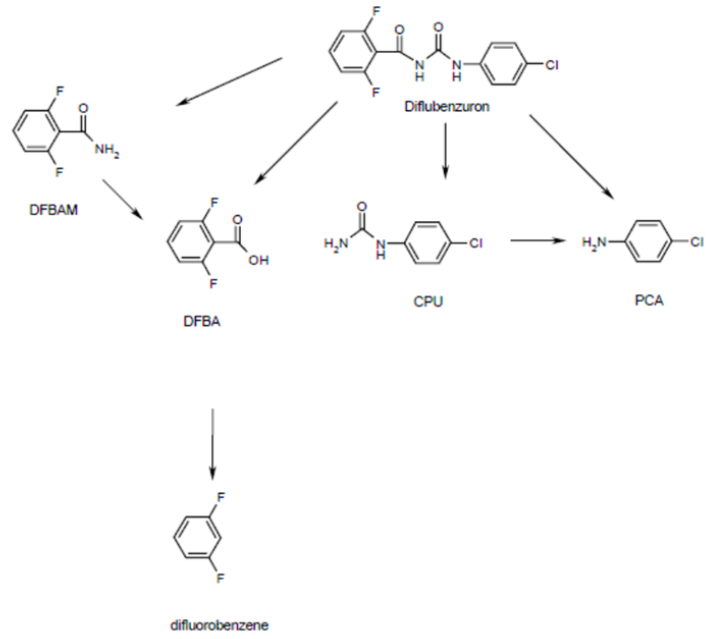

Fig. 1. Diflubenzuron (DFB) e produtos da sua degradação em água e solo. DFBA= ácido 2,6-difluorobenzóico, DFBAM= 2,6-difluorobenzamida, CPU= p-clorofeniluréia, PCA= p-cloroanilina (Nimmo et al., 1984).

As anilinas cloradas, como a PCA, além de serem produtos de degradação de pesticidas dos grupos fenil uréia e fenil carbamatos, também são compostos utilizados na produção de borrachas, plásticos, espuma de poliuretano, pigmentos e produtos farmacêuticos (Kataoka, 1996; Könnecker, G. Boehncke, A. Schmidt, 2003; Sihtmäe et al., 2010). Devido a sua importância toxicológica, a PCA e outras aminas substituídas foram incluídas pela Comunidade Européia na lista de poluentes industriais que devem ser monitorados em corpos d'água (Chhabra et al., 1991; Ekici et al., 2001). Considerada estável à hidrólise em relação a outras aminas, a PCA em meio aquoso a 35±1°C, pH 6.9 e na presença de lodo ativado apresentou meia-vida >100 horas, e há indícios de que sua meia-vida aumenta confome a

quantidade de matéria orgânica no meio (Ekici et al., 2001; Könnecker, G. Boehncke, A. Schmidt, 2003).

A p-cloroanilina foi classificada pela Agência de Proteção Ambiental dos Estados Unidos (E.P.A.) como mutagênica e provável carcinogênica para os seres humanos, devido à possibilidade destas aminas se converterem em compostos nitrosos quando entram em contato com a hemoglobina (Rodriguez et al., 1998). Além disso, seus metabólitos, 4-cloronitrosobenzeno e 4-cloronitrobenzeno, podem induzir tumores em órgãos como fígado, baço e bexiga (Burkhardt-Holm et al., 1999).

Lixiviados de áreas agrícolas, efluentes industriais e da piscicultura podem conter DFB e PCA que, se atingirem o ambiente aquático, podem sofrer misturas complexas entre si e outros xenobióticos e provocar impactos significativos em espécies sensíveis e não-alvo (Pérez et al., 2013).

Apesar do fenômeno de interações químicas ser conhecido há tempos, poucos são os estudos já realizados que privilegiam a observação dos efeitos tóxicos decorrentes da exposição a duas ou mais substâncias (Pérez et al., 2013). Em relação ao conceito de toxicidade de misturas, podem ocorrer interações como aditividade, sinergismo e antagonismo (Green and Abdelghani, 2004; Nair et al., 2007). Assim, a aditividade refere-se a dois agentes químicos que atuam independentemente sobre o mesmo sistema biológico de tal maneira que o efeito resultante é aditivo, ou a soma dos efeitos. O sinergismo é definido como uma interação entre os agentes tóxicos que produz um efeito maior que o esperado em relação às ações individuais, ou seja, maior que o efeito aditivo. Contrariamente a isto, agentes antagonistas reduzem o efeito, ou seja, produzem um efeito menor que o aditivo (Belden et al., 2007; Jonsson and Aoyama, 2007).

Outro importante fator a ser considerado na avaliação toxicológica dos xenobióticos nos ambientes aquáticos é a presença de solo, dada a sua capacidade em acumular compostos orgânicos e inorgânicos, principalmente por processos de decantação (Samuelsen et al., 2015). No entanto, uma vez no ambiente aquático, os contaminantes podem associar-se às partículas dos solo, ou podem permanecer na coluna d'água (Macken et al., 2015; Samuelsen, 2016). Essa partição depende das propriedades físicas e químicas da água, do tipo de solo presente e da natureza molecular do xenobiótico (Hartman e Martin, 1984; Jonsson e Nunes Maia, 1999). Assim, esta partição pode afetar a disponibilidade dos xenobióticos para diferentes organismos aquáticos (Martins et al., 2012; Samuelsen, 2016).

Não há na literatura dados acerca da toxicidade da mistura do DFB e PCA e a maioria das pesquisas que são realizadas com os compostos individualmente não levam em

consideração a presença do solo (Macken et al., 2015; T Scholz, 1999; Zaidi et al., 2013). Diante do exposto a completa elucidação dos efeitos adversos da mistura destes compostos, na presença e ausência de solo, a organismos ecologicamente relevantes faz-se extremamente necessária para avaliação de risco ambiental, direcionamento das políticas públicas e determinação de limites permissíveis.

Os peixes são incessantemente expostos a misturas de xenobióticos ao longo da sua vida e são muito vulneráveis aos efeitos dos poluentes por possuírem a pele e as brânquias altamente permeáveis, tornando-se orgânismos relevantes para nossos estudos (Di Giulio and Hinton, 2008). Bioensaios com peixes permitem estudar, em condições controladas, alguns parâmetros como mortalidade, alterações no desenvolvimento, no comportamento e danos nos tecidos ou células, podendo ajudar a predizer alguns efeitos de contaminantes em ecossistemas aquáticos naturais (Carvalho, 2012). Esses organismos são considerados padrões para testes de toxicidade aguda, assim como para testes de toxicidade crônica e a sua importância em ecotoxicologia é tanto ecológica quanto econômica. O fato dos peixes ocuparem níveis tróficos elevados entre os organismos aquáticos faz com que estes animais, através da cadeia alimentar, acumulem altos teores de substâncias por biomagnificação. Além disso, os peixes podem ser considerados a principal rota de contaminação humana (Authman et al., 2015; Schreiner et al., 2016).

Portanto, este trabalho analisou os efeitos tóxicos das misturas de DFB com PCA, nas proporções de 75%, 50% and 25% de PCA, nos peixes tilapia *(Oreochromis niloticus)* e zebrafish *(Danio rerio)* na presença e ausência de solo.

Os peixes *Oreochromis niloticus*, popularmente conhecidos como tilápia, (Figura 2), são espécies originárias dos rios e lagos africanos que ganharam cada vez mais espaço comercial no mundo por ser de fácil criação e pouco onerosa. A alta qualidade de sua carne faz deste peixe um produto de interesse industrial e de boa aceitação pelo mercado consumidor (F.A.O., 2016). Com o desenvolvimento significativo da sua criação e a falta de manejo correto e sustentável veio o aumento das perdas ocasionadas pelas parasitoses. Devido à utilização do DFB nos tanques de criação das tilápias para minimizar estas perdas, esta espécie de peixe foi escolhida para nossos estudos. Além de que, testes de toxicidade e bioquímicos mostraram que esta espécie é sensível a uma grande variedade de compostos e pode ser considerada como um potencial bioindicador (Carvalho et al., 2012; Fırat et al., 2011; Jonsson et al., 2015; Meng et al., 2013)

Fig. 2. *Oreochromis niloticus*, popular tilápia (Foto: Dantzger, 2016).

Danio rerio, conhecido popularmente como zebrafish ou paulistinha (Figura 3), é uma espécie de peixe ovípara de origem da Africa do Sul, membro da família Cyprinidae e possui um tempo de vida médio de três a cinco anos (Nagel, 2002).

Fig. 3. *Danio rerio adulto*, popular zebrafish (Foto: Dantzger, 2016).

O zebrafish tem sido um importante modelo de vertebrado para diversos estudos toxicológicos desde 1930 e seu embrião foi extensamente estudado (Kimmel et al., 1995) tornando-se um modelo bastante utilizado em pesquisas de diversas áreas.

Estes peixes são fáceis de reproduzir e os custos com sua criação são relativamente baixos. São organismos que, quando adultos, medem cerca de 4 a 5 cm de comprimento e atingem a maturidade sexual após 3 ou 4 meses de vida. Cada fêmea de zebrafish pode colocar cerca de 200 a 300 ovos por semana e os embriões se desenvolvem rapidamente com embriogênese completa, cinco dias após a fertilização (Zhang et al., 2003). Os embriões (Figura 4A) e as larvas (Figura 4B) destes peixes são transparentes, portanto, estruturas morfológicas e órgãos internos como cérebro, olhos, coração, fígado e rim podem ser facilmente visualizados utilizando microscopia óptica, sem necessidade de cirurgia. (Parng et al., 2002).

Devido ao seu pequeno tamanho, um único embrião ou larva pode ser mantido em volumes de fluidos pequenos e pode ser criado em poços individuais de microplacas (Parng et al., 2002).

Bioensaios que utilizam embriões de peixe estão sendo cada vez mais empregados no lugar dos testes que utilizam peixes adultos e modelos de mamíferos (Lin et al., 2013). Os testes com embriões têm uma forte correlação com os resultados de testes de toxicidade aguda que utilizam peixes adultos e é provável que os embriões não tenham a mesma percepção de dor que os adultos, devido à imaturidade do sistema nervoso (Lammer et al., 2009).

Os ensaios de toxicidade com embrião do zebrafish permitem o monitoramento de uma ampla gama de endpoints que podem esclarecer os efeitos dos produtos químicos sobre, por exemplo, batimentos cardíacos, pigmentação ou desenvolvimento dos olhos e do cérebro. O teste pode potencialmente ser prolongado até 96 h ou até mesmo por 120 h para detectar efeitos em endpoints subletais adicionais, tais como, taxa de eclosão e anormalidades larvais (OECD, 2013).

Além de todas as vantagens descritas acima, o zebrafish foi escolhido como organismos teste deste trabalho por compartilhar muitas características biológicas, fisiológicas e comportamentais com os seres humanos (McGrath and Li, 2008; Parng et al., 2002). Os genes do zebrafish são, em média, 75% homólogos aos genes humanos e ortólogos para alguns genes que são conhecidos por desempenhar papéis fundamentais em doenças humanas (Barbazuk et al., 2000).

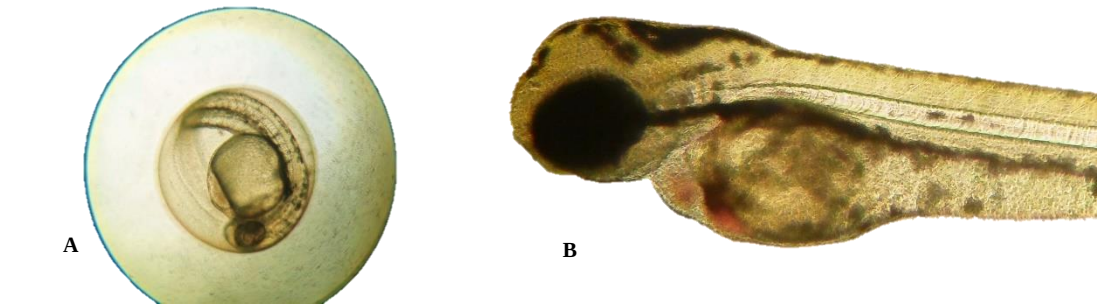

Fig. 4. (A) Embrião de zebrafish com 72 horas pós-fertilização (B) Larva de zebrafish com 72 horas pós-fertilização. Aumento de 40 vezes em microscópio invertido (Fotos: Dantzger, 2016).

A exposição do zebrafish e da tilapia a contaminantes como DFB e PCA pode causar alterações biológicas nestes organismos. Estas alterações podem ser medidas e utilizadas como biomarcadores que permitem a rápida avaliação da saúde dos organismos e alertar sobre as possíveis alterações ambientais (Serafim et al., 2012).

Alterações a nível bioquímico ou molecular são normalmente as primeiras respostas detectáveis e quantificáveis em uma mudança do meio ambiente. Variações nos sistemas

bioquímicos são frequentemente indicadores mais sensíveis que os de maiores níveis de organização biológica, tais como células, organismos e populações (van der Oost et al., 2003). Por causa destas características, os indicadores bioquímicos são apontados como sistemas de "sinal de alerta" na avaliação da saúde ambiental (Pereira Maduenho and Martinez, 2008).

Para a verificação de contaminação dos corpos hídricos por compostos químicos, como o DFB e PCA, a utilização de indicadores bioquímicos pode ser mais vantajosa economicamente quando comparado à implementação de protocolos de análise que utilizam métodos de alto custo e que demandam maior tempo de análise (Serafim et al., 2012).

Os indicadores bioquímicos escolhidos para a realização deste estudo foram as enzimas fosfatases ácida (AcP) e alcalina (AlP), catalase (CAT) e as transaminases (alanina (ALT) e aspartato (AST) aminotransferases) extraídas dos fígados e brânquias das tilápias. Estas enzimas foram selecionadas com base na capacidade de cada uma responder à exposição aos contaminantes.

As fosfatases são enzimas hidrolíticas que catalisam a desfosforilação de uma grande variedade de ésteres de ortofosfato e reações de transfosforilação (Aoyama et al., 2003). Estas enzimas são encontradas em animais, vegetais e microrganismos e estão associadas a diversos processos metabólicos, bem como ao crescimento e diferenciação celular. A fosfatase ácida (AcP) é uma hidrolase lisossomal que, em situações de estresse celular, pode extravasar para fluidos intra e extracelulares, além de participar do processo de autólise celular (Kumaresan and Karuppasamy, 2011; Suresh et al., 1993). A fosfatase alcalina (AlP) é uma enzima polifuncional ligada à membrana (Coleman, 1992; Molina et al., 2005) que desempenha funções importantes como a regulação da síntese protéica, a atividade secretora, a espermatogênese, o metabolismo do glicogênio e as atividades de transporte da membrana (Thirumavalavan, 2010). Assim, qualquer alteração na atividade dessas enzimas pode afetar a saúde de um organismo de alguma forma (Fırat et al., 2011; Palanivelu et al., 2005).

As enzimas catalase (CAT) fazem parte dos sistemas antioxidantes dos seres vivos pela capacidade de neutralizar a ação de espécies reativas de oxigênio (EROs), como do ânion superóxido ($O_2^{\bullet-}$) e do peróxido de hidrogênio (H_2O_2). Estas moléculas, altamente reativas, fazem parte de subprodutos do metabolismo respiratório normal e desempenham papel importante nos processos de sinalização celular. Entretanto, em situações de estresse celular, as EROs podem aumentar significativamente e causar danos celulares, como alteração do DNA, peroxidação de lipídeos e oxidação de aminoácidos nas proteínas (Fridovich, 1995; Oruç and Uner, 2000). A CAT está presente nos peroxissomos de quase todas as células aeróbias e a sua atividade pode aumentar ou diminuir conforme a produção de H_2O_2 em organismos expostos a

poluentes. Dessa forma a avaliação de sua atividade pode ser realizada através de métodos fáceis, validados e de baixo custo. Por esse motivo, geralmente, a catalase é utilizada em estudos para verificação de estresse oxidativo (Thirumavalavan, 2010). A alteração da atividade desta enzima extraída de organismos expostos a poluentes químicos de diversas origens tem sido alvo de estudo na análise de risco e monitoramento de áreas degradadas (Hamed et al., 2016). .

As transaminases, aspartato aminotransferase (AST) e alanina aminotransferase (ALT), são enzimas envolvidas no metabolismo de aminoácidos que permitem a transferência de grupos amina de aminoácidos para cetoácidos, em reações designadas de transaminação. Estas enzimas são intracelulares e encontradas predominantemente em hepatócitos, embora a AST também seja encontrada em outros tecidos como, coração, glóbulos vermelhos e músculo e alterações em suas atividades podem permitir a identificação de danos nestes órgãos (Poelzl et al., 2012).

OBJETIVOS

O objetivo geral deste trabalho foi avaliar os efeitos tóxicos dos compostos, DFB e PCA, individualmente e em diferentes proporções de suas misturas, na presença e ausência de solo, nos peixes tilápia e zebrafish. Os objetivos específicos alcançados foram:

- Determinação da concentração letal média do DFB e PCA (CL50-96h) individualmente e de suas misturas para os peixes, tilápia e zebrafish, na presença e na ausência de solo;

- A partir dos valores de CL50-96h, foram avaliados a capacidade de interação dos compostos com o solo e o tipo de interação entre suas misturas;

- Avaliação dos efeitos tóxicos de concentrações subletais dos compostos e suas misturas nas tilápias por meio da determinação da atividade das enzimas: fosfatase ácida (AcP) e alcalina (AlP), catalase (CAT), aspartato aminotransferase (AST) e alanina aminotransferase (ALT).

- Verificação da toxicidade dos compostos e suas misturas para os embriões e larvas de zebrafish através de ensaios para obtenção da taxa de mortalidade e de eclosão, malformação embrionária e alterações morfológicas e histológicas.

Assim, esta tese foi estruturada em 3 capítulos cada um apresentando um artigo submetido em periódicos científicos internacionais.

O capítulo 1 apresentará a avaliação da toxicidade aguda do DFB, PCA e suas misturas, na presença e ausência de solo, para as tilápias e os resultados obtidos com as enzimas biomarcadoras extraídas do fígado destes organismos. O capítulo 2 discorre sobre o tipo de interação que ocorre entre as misturas do DFB e PCA, a presença de solo nos testes e a alteração das enzimas extraídas das brânquias das tilapias. Por fim, o capítulo 3 abrange a investigação da toxicidade dos compostos e suas misturas sobre os embriões, larvas e adultos do zebrafish.

A metodogia utilizada neste trabalho, além de ser descrita nos artigos que compõe os capítulos desta tese, será mais bem detalhada a seguir.

MATERIAIS E MÉTODOS

1. Compostos Avaliados

Foram avaliados o inseticida Diflubenzuron (DFB), presente na formulação comercial Dimilin, tipo pó molhável (250 g/Kg, Chemtura Indústria Química do Brasil Ltda) e seu metabólito p-cloroanilina (PCA) (99% pureza, Sigma-Aldrich).

As soluções teste do DFB, PCA e suas misturas foram preparadas com água declorinizada para uso imediato, não havendo o armazenamento destas.

2. Solo Artificial

O solo artificial utilizado nos testes foi preparado através de uma mistura que continha:

- 70% areia fina autoclavada - cedida pelo Instituto de Pesquisas Tecnológicas de São Paulo (IPT);

- 20% caulim (CAS Number: 1332-58-7 Sigma-Aldrich);

- 10% turfa autoclavada - comprada da Mineração Darcy, São Simão, SP, Brasil.

O solo (Figura 5) foi preparado de acordo com o protocolo 207 da Organização para Cooperação e Desenvolvimento Econômico (OECD, 1984).

Fig. 5. Solo artificial preparado para utilização nos testes ecotoxicológicos com DFB e PCA.

3. Obtenção e condições de cultivo do peixe tilápia *(Oreochromis niloticus)*

As tilápias foram adquiridas da Piscicultura Polletini, localizada na cidade de Mogi Mirim, SP. Os peixes foram aclimatados no Laboratório de Enzimologia do Departamento de Bioquímica e Biologia Tecidual, Instituto de Biologia, UNICAMP, Campinas, SP.

A aclimatação dos peixes foi realizada de acordo com a norma da OECD 203 (OECD, 1992), em condições de temperatura controlada, 28 ± 2°C e fotoperíodo natural, por 4 semanas antes do início dos testes. Durante esse tempo os animais foram mantidos em tanques plásticos de 175 litros (Figura 6) com água declorinizada (filtrada), aeração constante e foram alimentados com a ração comercial Tetramin (fornecida 2 vezes ao dia). Os parâmetros físicos e químicos da água foram monitorados e permaneceram constantes: pH 7,4 ± 0,4; oxigênio dissolvido 8 ± 2 mg O_2/L; condutividade 160 ± 5 μS/cm e dureza 50 ± 2 mg $CaCO_3$/L.

Nos testes em que houve a necessidade do sacrifício dos peixes o mesmo foi realizado com anestesia, (benzocaína 0,1 g/L), seguida da secção medular sem provocar qualquer sofrimento aos animais. Os peixes, após o sacrifício, foram recolhidos por uma empresa terceirizada que fez o descarte correto dos mesmos.

Fig. 6. Aclimatação das tilápias em tanques plásticos com capacidade para 175 litros de água filtrada, aeradores e filtros. Temperatura da água mantida por termostatos a 28 + 2ºC sob luminosidade natural da sala.

4. Obtenção e condições de cultivo do peixe zebrafish *(Danio rerio)*

O zebrafish foi obtido de um fornecedor local (Agrosete, Sumaré, SP) e a sua criação foi estabelecida no Laboratório de Enzimologia no Departamento de Bioquímica e Biologia Tecidual, Instituto de Biologia, UNICAMP, Campinas, SP.

Os peixes adultos foram mantidos em aquário de vidro contendo 10L de água filtrada a 28 ± 2ºC. Os parâmetros físicos e químicos da água foram monitorados e permaneceram constantes: pH 7,4 ± 0,4; oxigênio dissolvido 8 ± 2 mg O_2/L; condutividade 160 ± 5 µS/cm e dureza 50 ± 2 mg $CaCO_3$/L (Figura 7).

Os peixes foram expostos a um ciclo natural de iluminação e alimentados duas vezes ao dia, com alimento comercial TetraMin Plus (Tetra Holding US Inc.) e uma vez com artemias. Estes organismos foram divididos em 2 grupos: um que foi mantido como matriz para obtenção de embriões/larvas e outro que foi utilizado nos testes de toxicidade aguda.

Todos os experimentos com os peixes (tilápias e zebrafish) foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas-CEUA/UNICAMP **(Protocolo n°3641-1. Ver ANEXO 1).**

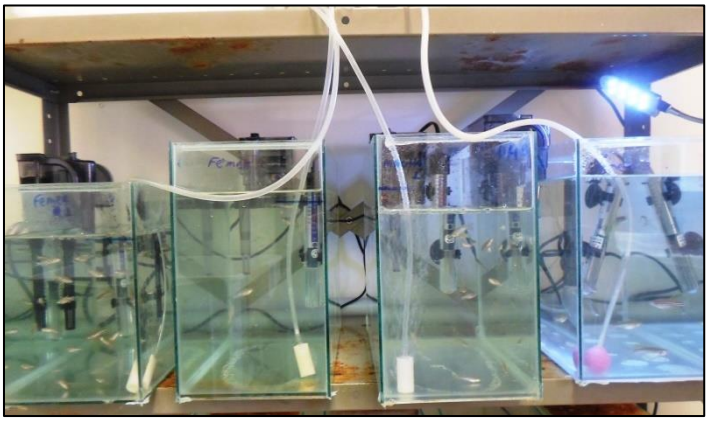

Fig. 7. Criação de zebrafish em aquários contendo 10 litros de água filtrada, aeradores e filtros. Temperatura da água mantida por temostatos a 28 + 2ºC sob luminosidade natural da sala.

5. Testes de toxicidade aguda com os peixes: tilápia e zebrafish adultos

Após o período de aclimatação foi realizado o teste de toxicidade aguda com os peixes adultos de tilápias e zebrafish para obtenção da concentração letal média (CL50-96h) dos compostos individualmente e das misturas de DFB com 75%, 50% e 25% de PCA. Essas proporções dos compostos nas misturas foram escolhidas para que no estudo do modelo de predição de toxicidade de misturas fosse coberta uma maior superfície de concentração-resposta (Syberg et al., 2008). As concentrações avaliadas de diflubenzuron, p-cloroanilina e suas misturas para as tilápias foram: 0 (controle) - 0,1 - 10 e 100 mg/L e para o zebrafish foram: 2.2, 4.4, 9.7, 21.3, 46.8 e 100 mg/L. A escolha dessas concentrações foi baseada em pesquisas publicadas de CL50-96h dos mesmos compostos para essas espécies de peixes (Burkhardt-

Holm et al., 1999b; Jonsson et al., 2015). O ensaio foi realizado em duplicata e na presença e ausência de solo.

Para a realização dos testes, 10 peixes por concentração avaliada dos compostos e suas misturas foram transferidos para aquários de 10 litros, dotados de aeração artificial e termostatos que mativeram a temperatura da água constante (28 ± 2 °C). Para os testes com solo, 900 gramas deste foram vigorasamente misturadas com a solução teste dentro de cada aquário antes da transferência dos peixes. A quantidade de solo foi escolhida com base em uma pesquisa de toxicidade aguda com DFB e solo (Medeiros et al., 2013). O sistema utilizado neste ensaio foi o estático devido à estabilidade dos compostos nas condições físico-químicas da água e o tempo de realização do teste (Ekici et al., 2001; Zaidi et al., 2013). Os parâmetros físico-químicos da água dos aquários foram monitorados e permaneceram constantes: pH 7,4 ± 0,4; oxigênio dissolvido 8 ± 2 mg O_2/L; condutividade 160 ± 5 μS/cm e dureza 50 ± 2 mg $CaCO_3$/L.

O período total de exposição dos peixes aos compostos químicos foi de 96 horas, durante as quais, os organismos não foram alimentados de acordo com o protocolo 203 da OECD (OECD, 1992). Após as 96 horas de teste, os indivíduos mortos foram contados e os dados obtidos foram analisados estatisticamente para a obtenção da concentração que foi letal para 50% dos peixes.

6. Modelo para predição de toxicidade das misturas de DFB e PCA

Na avaliação dos tipos de interação entre as misturas de DFB e PCA foram utilizados os valores de CL50-96h obtidos paras as tilápias, na ausência e presença de solo. O procedimento foi baseado no método proposto por Marking (1977) e consistiu em obter o índice de toxicidade aditivo (AI) da mistura a partir da soma da toxicidade individual dos compostos, utilizando-se a seguinte equação:

$$S = \frac{Am}{Ai} + \frac{Bm}{Bi}$$

Onde: S = soma da toxicidade individual dos compostos; A e B correspondem aos produtos químicos, neste caso DFB e PCA; i e m correspondem aos valores de CL50-96h dos produtos químicos isolados e na mistura, respectivamente.

Os valores dos índices de adição AI foram calculados das seguintes equações:

$$AI = \frac{1}{S} - 1 \quad \text{Quando } S \leq 1$$

$$AI = S(-1) + 1 \quad \text{Quando } S \geq 1$$

Se o valor de AI for 0, um efeito aditivo simples é diagnosticado; para AI < 0, o efeito é antagônico e para AI > 0, o efeito é considerado sinérgico.

Após a obtenção dos valores de AI, foi calculado o Fator de Magnificação (MF) que demonstra quantas vezes a mistura é mais ou menos tóxica do que os compostos isolados. O valor de MF é calculado pela adição de 1 ao valor de AI, no caso de sinergismo e, no caso de antagonismo, o valor de MF é obtido através do inverso do valor absoluto de AI.

O isobolograma foi utilizado para mostrar graficamente a interação toxicológica (sinergismo, aditividade ou antagonismo) para todas as misturas binárias testadas de DFB e PCA. Este gráfico foi construído traçando-se as concentrações dos poluentes que, isoladamente e, em combinação, induziram 50% de letalidade dos peixes (CL50-96h) (Jonsson and Aoyama, 2007; Marking, 1977; Rand and Petrocelli, 1985).

7. Ensaio com tilápias *(Oreochromis niloticus)* para avaliação dos biomarcadores enzimáticos

Para a avaliação da atividade das enzimas AcP, AlP, CAT, ALT e AST, 10 tilápias de 3 a 4 cm de comprimento (Figura 8A) foram expostas às concentrações subletais de DFB, PCA e suas misturas, na presença e ausência de solo.

As concentrações subletais testadas foram calculadas a partir dos valores de CL50-96h dos compostos e de suas misturas obtidos nos testes de toxicidade aguda (tabela 1).

Tabela 1. Concentrações subletais de DFB, PCA e suas misturas em mg/L testadas nos ensaios bioquímicos.

Condições	DFB	PCA	75%PCA	50% PCA	25%PCA
Sem solo	1-2-10	0.25 - 0.5 - 2.5	0.15 - 3 - 1.5	0.4 - 0.8 - 4	1- 2 - 10
Com solo	1-2-10	0.37 - 0.74 -3.7	0.25 - 0.5 - 2.5	0.45 - 0.9 - 4.5	1 - 2 - 10

Concentrações subletais foram equivalentes a CL50/10, CL50/50 e CL50/100.

Os organismos foram expostos aos compostos em aquários de vidro (Fig. 8B) contendo 10 litros de solução teste, aeração constante, luminosidade natural e temperatura

controlada (28 ± 2ºC). Para os testes na presença de solo os procedimentos foram os mesmos descritos anteriormente no item 5.

Os parâmetros físico-químicos da água dos aquários foram monitorados e permaneceram constantes: pH 7,4 ± 0,4; oxigênio dissolvido 8 ± 2 mg O_2/L; condutividade 160 ± 5 µS/cm e dureza 50 ± 2 mg $CaCO_3$/L.

A duração do ensaio foi de 96 horas durante as quais os peixes permaneceram sem alimentação de acordo com o protocolo 203 da OECD (OECD, 1992). Após este período os organismos foram coletados, anestesiados com 800 µL de benzocaína, com concentração de 0,1g/L, em 1 L de água, e sacrificados através de secção medular. O fígado e as brânquias foram retirados e, em seguida, utilizados para preparação de extratos para dosagens enzimáticas (Figura 8C). Do fígado dos peixes foram dosadas as enzimas AcP, AlP, CAT, AST e ALT e das brânquias as mesmas enzimas foram dosadas com exceção das transaminases.

Os órgãos foram divididos em partes iguais, pesados e homogeneizados em tampão apropriado para cada enzima na proporção de 1:4 (massa/volume). Após, foi realizada a centrifugação do homogeneizado a 10.000 r.p.m. na centrífuga Eppendorf 5810R v8.2, com o rotor FA-45-30-11, por 10 minutos, onde se obteve o sobrenadante contendo as enzimas que foram analisadas nos testes bioquímicos (Figura 8D).

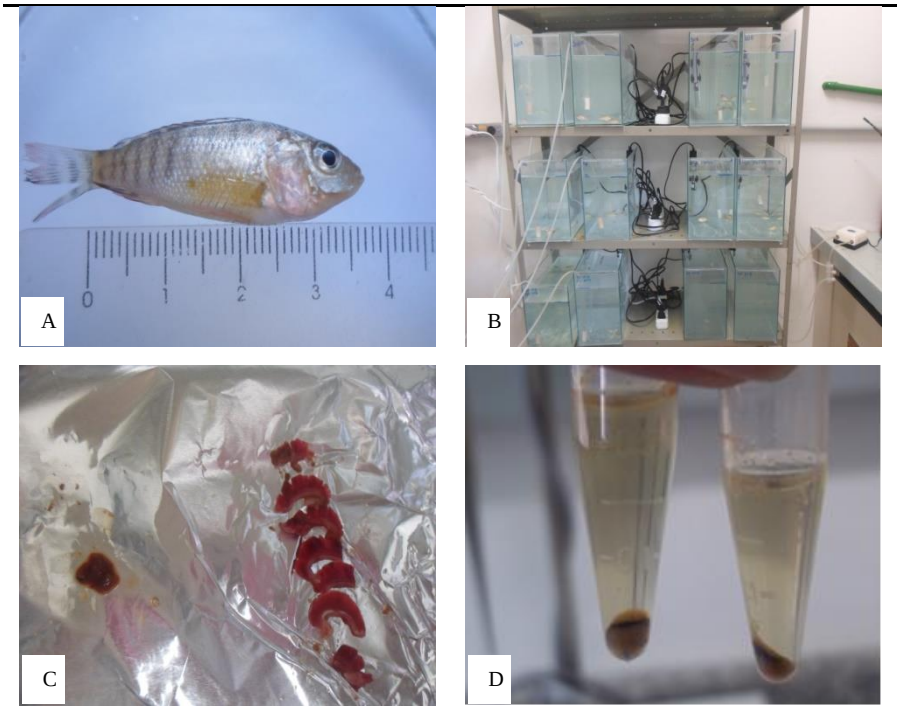

Fig. 8. (A) Tilápia (3,5 cm de comprimento) utilizada na avaliação dos biomarcadores enzimáticos. (B) Aquários contendo 10 litros de soluções testes, n=10 peixes (5 em cada réplica), aeradores, filtros e termostatos (C) Fígado

e brânquias retirados após a exposição dos peixes aos compostos. (D) Tubos com sobrenadante contendo as enzimas que foram analisadas.

7.1 Determinação da atividade das enzimas fosfatases ácida (AcP) e alcalina (AlP)

As atividades das enzimas AcP e AlP foram determinadas em microplacas de 96 poços. Para a enzima AcP, foram adicionados 10 µL do sobrenadante contendo a enzima, 15 µl de tampão de acetato 100 mM (pH 5) e 125 µl de solução de p-nitrofenilfosfato (pNPP) 10 mM. A atividade de AlP foi medida como a da AcP, com excepção da adição de 15 µL de tampão de glicina 250 mM (pH 9.4) e 10 µL de $MgCl_2$ 20 mM como co-fator. As misturas foram incubadas durante 40 min. a 37 °C, em seguida esta reação foi interrompida pela adição de 50 µL de NaOH 1 M, seguido pela medição da absorbância a 405 nm, devido à formação de p-nitrofenóxido (pNP) de coloração amarela (coeficiente de extinção molar = 18.000 $M^{-1}cm^{-1}$) (Jonsson and Aoyama, 2007). As atividades específicas da AcP e AlP foram expressas em nmoles $pNP.min^{-1}.mg\ proteína^{-1.}$ As análises foram realizadas em triplicata em um espectrofotômetro de microplacas EON BioTek.

7.2 Determinação da atividade da enzima catalase (CAT)

A atividade da enzima CAT foi determinada em microplacas de 96 poços apropriadas para leitura em espectofotômetros com comprimentos de ondas em U.V. Para isso foram adicionados 50 µL do sobrenadante contendo a enzima em 250 µL de peróxido de hidrogênio (substrato) 0,03 M preparado em tampão fosfato 50 mM a pH 7. Em seguida, a absorbância a 240 nm foi monitorada durante 1 min no espectrofotômetro de microplacas EON BioTek. A atividade específica de CAT (nmoles de $H_2O_2.min^{-1}.mg\ proteína^{-1}$) foi determinada de acordo com o método descrito por Aebi (Aebi, 1984).

7.3 Determinação da atividade das enzimas aspartato (AST) e alanina (ALT) aminotransferase

A atividade da aspartato (AST) e alanina (ALT) aminotransferase em homogenato de fígado foram medidas em microplacas de 96 poços utilizando kits comerciais Bioliquid, obtidos da Laborclin (São Paulo, Brasil). A enzima AST produz oxaloacetato que é reduzido a malato pela enzima malato desidrogenase na presença de NADH. A diminuição de NADH foi medida a 340 nm no espectrofotômetro de microplacas EON BioTek e foi proporcional à atividade da enzima AST. O piruvato produzido pela ALT é reduzido a lactato pela enzima

lactato desidrogenase, na presença de NADH, cuja diminuição é proporcional à atividade da ALT. A diminuição de NADH foi medida a 340 nm no espectrofotômetro de microplacas EON BioTek e foi proporcional à atividade da enzima ALT. Ambas as desidrogenases estão presentes com os substratos no kit comercial. De acordo com o kit, as atividades enzimáticas foram expressas em U/mL.

8. Determinação da concentração de proteínas

Para a determinação da atividade específica das enzimas (atividade enzimática/mg de proteína), a proteína do sobrenadante foi quantificada pelo método de Lowry (Lowry et al., 1951) a 560 nm no espectrofotômetro de microplacas EON BioTek, utilizando albumina de soro bovino como padrão.

9. Obtenção dos embriões de zebrafish *(Danio rerio)*

Os machos e as fêmeas de zebrafish que conviviam no mesmo aquário, foram separados três dias antes do acasalamento permanecendo os machos em um aquário e as fêmeas em outro (proporção de 2 machos para 1 fêmea). No dia anterior ao acasalamento, estes peixes foram transferidos para outro aquário que continha uma divisória com furos que permitiam a passagem de água de um lado para o outro do mesmo. Assim, os machos permaneceram de um lado da divisória e as fêmeas do outro. Este aquário de acasalamento, além da divisória, foi forrado por bolinhas de vidro (populares bolinhas de gude) que serviram de proteção aos ovos contra a predação dos próprios peixes (Figura 9A).

O estímulo luminoso faz com que ocorra a postura dos ovos e aconteça a cópula. Para isso, o aquário de acasalamento permaneceu *overnight* no escuro, com termostato e filtro mantendo os parâmetros físico-químicos da água constantes: pH 7,4 ± 0,4; temperatura = 28 ± 2ºC, oxigênio dissolvido 8 ± 2 mg O_2/L; condutividade 160 ± 5 μS/cm e dureza 50 ± 2 mg $CaCO_3$/L.

No dia do acasalamento, o filtro do aquário foi desligado, a divisória retirada e as luzes do biotério foram acesas. Os peixes permaneceram no ato de acasalamento por aproximadamente 40 minutos. Após esse período, os peixes foram retirados do aquário de acasalamento e retornaram para os aquários de convívio diário. As bolinhas de vidro foram cuidadosamente recolhidas e a água do aquário onde ocorreu o acasalamento passou por uma peneira onde os ovos ficaram retidos.

Com o auxílio de um estereomicroscópio e pipetas plásticas do tipo Pasteur, os ovos foram transferidos para uma placa de Petri contendo água filtrada, a mesma utilizada na de criação dos peixes adultos. Em seguida, os ovos coagulados (Figura 9B) ou não fertizados foram descartados e os ovos fecundados (Figura 9C) foram transferidos para as condições de teste descritas a seguir.

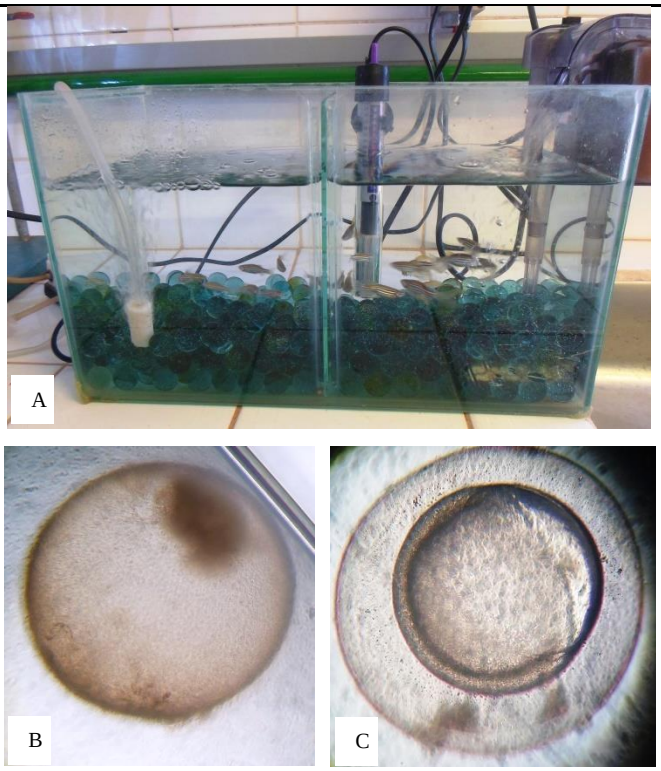

Fig. 9. (A) Machos e fêmeas adultos de zebrafish em aquário de acasalamento com divisória perfurada, bolinhas de vidro, aeração, filtro e termostato. (B) Ovo coagulado descartado. (C) Ovo fertilizado em processo de divisão utilizado nos testes. Imagens obtidas no microscópio invertido Nikon Eclipse TS100 com aumento de 10 vezes.

9.1 Ensaio para observação do desenvolvimento dos embriões de zebrafish expostos aos compostos e suas misturas

Foram utilizados 48 embriões de zebrafish, com 1 hora pós-fertilização (1hpf), por concentração testada dos compostos e suas misturas. Estes embriões foram distribuídos individualmente em microplacas de 24 poços com 2 ml de solução teste em cada poço (20 embriões por microplaca e duas réplicas por concentração). Em cada microplaca de 24 poços, quatro deles foram destinados para o controle interno contendo 1 embrião por poço com 2 mL de água filtrada (Figura 10). Os embriões foram expostos por 96 horas às seguintes concentrações de diflubenzuron, p-cloroanilina e suas misturas: 2.2, 4.4, 9.7, 21.3, 46.8 e 100 mg/L. As misturas de DFB seguiram as proporções de 75%, 50% e 25% de PCA.

As soluções teste foram preparadas com água declorinizada (pH 7,00 ± 0,5 e condutividade 160 ± 5 μS cm^{-1}) e as microplacas com os embriões foram colocadas em estufa germinadora Eletrolab que manteve a temperatura constante de 28 ± 2ºC e um fotoperíodo de 12 horas luz e 12 horas escuro.

As alterações no desenvolvimento embrionário foram observadas a cada 24 horas em microscópio invertido (Nikon Eclipse TS100) e estereomicroscópio Bel photonics®. Alterações (endpoints) como coagulação de ovos, mortalidade (falta de desenvolvimento embrionário ou coagulação de material nuclear), edema pericárdico, edema do saco vitelino e malformação da espinha foram observadas e registradas através de uma câmera digital OptikamB3 acoplada ao microscópio e ao estereomicroscópio (Figura 11). O ensaio foi baseado na diretriz 203 da OCDE sobre o Teste de Toxicidade Embrionária de Peixe (FET) (OECD, 2013).

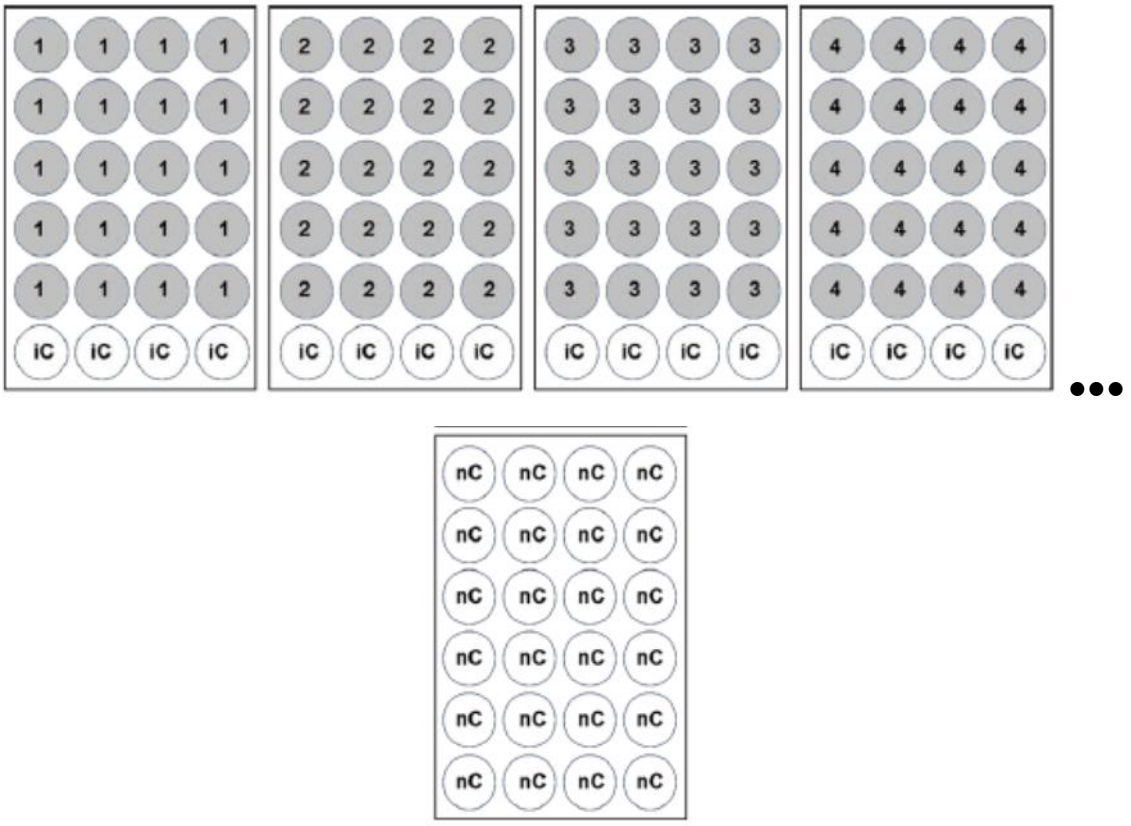

Fig. 10. Esquema das micloplacas de 24 poços utilizadas para a realização dos testes com embriões/larvas de zebrafish. 1-4= soluções teste com 1 embrião por poço (6 placas por teste), IC= controle interno com água declorinizada e 1 embrião por poço, nC= placa com embriões controle com água declorinizada (Ilustração adaptada do protocolo 203 da OECD).

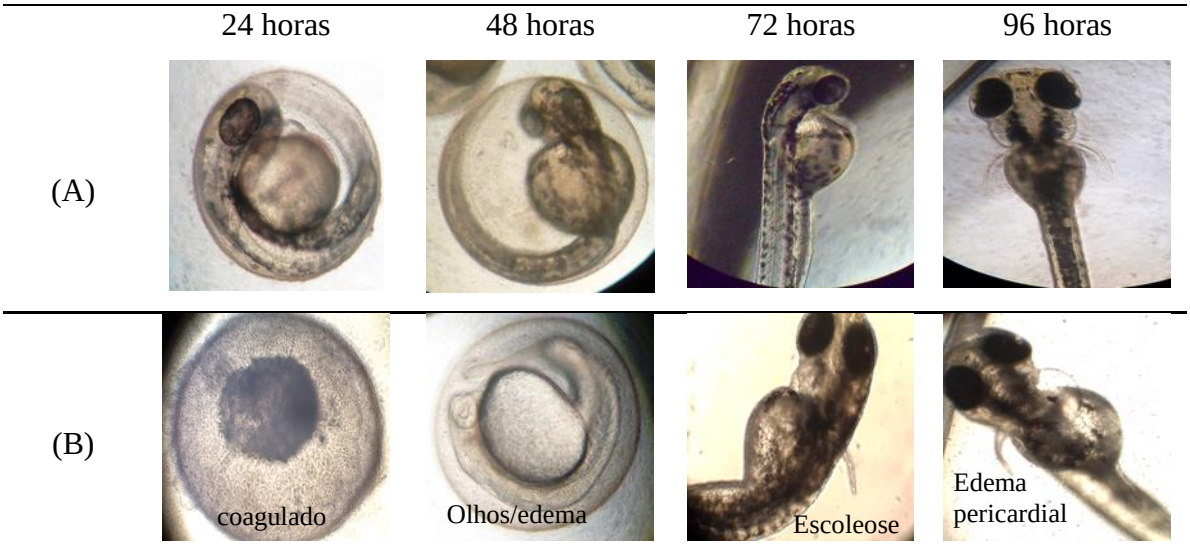

Fig. 11. Fotos de embriões/larvas de zebrafish com normal (A) e anormal (B) desenvolvimento.

9.2 Ensaio de toxicidade aguda com as larvas de zebrafish

Para o teste de toxicidade aguda com as larvas, 48 organismos com 96 horas após a fertilização (hpf) foram expostos aos compostos e suas misturas em duas microplacas de 24 poços sob as mesmas condições descritas no ensaio com os embriões do item 9.1**.** As concentrações de diflubenzuron, p-cloroanilina e suas misturas testadas foram: 2.2, 4.4, 9.7, 21.3, 46.8 e 100 mg/L. As misturas de DFB seguiram as proporções de 75%, 50% e 25% de PCA.

As soluções teste foram preparadas com água declorinizada (pH 7,00 ± 0,5 e condutividade 160 ± 5 μS cm-1) e as microplacas com as larvas foram colocadas em estufa germinadora Eletrolab durante o teste e a temperatura se manteve constante em 28 ± 2ºC e um fotoperíodo de 12 horas luz e 12 horas escuro.

Após o período total de exposição das larvas às concentrações teste dos compostos e suas misturas o número de organismos mortos foi registado para determinar CL50-96h.

9.3 Ensaios de morfologia e histologia com as larvas de zebrafish

Nos testes morfológicos, 10 Larvas com 144 hpf foram expostas às concentrações subletais do DFB, PCA e suas misturas por 96h. As concentrações subletais foram calculadas a partir da CL50-96h obtidas no teste de toxicidade aguda e divididas por 10 (Tabela 2). As misturas de DFB seguiram as proporções de 75%, 50% e 25% de PCA e o delineamento do ensaio foi o mesmo descrito no item 9.1.

Após 96h de exposição, as larvas foram fixadas vivas com goma de xantina 1% em lâminas de microscopia e alterações morfológicas no fígado, intestino e bexiga natatória em relação ao controle de larvas foram avaliadas através de microscópio invertido Nikon Eclipse TS100 com ampliação 40X e estereomicroscópio BEL photonics® com ampliação 2X (Zhang et al., 2003, McGrath e Li 2008).

Nos testes histológicos, 10 larvas com 144 hpf foram expostas à concentrações subletais (Tabela 2) do DFB, PCA e suas misturas por 96h. Após as 96h as larvas foram anestesiadas por uma solução de metanossulfonato de tricaína (MS-222, Sigma) a 0,3 g/L e colocadas em tubos de 2mL com paraformaldeído (4% v/v) preparado em tampão PBS 0,1M durante 24 horas a 4ºC. Após esse processo chamado de fixação, o paraformaldeído foi removido com cinco lavagens de 5 minutos em PBS 0,1 M (pH 7,4) e as larvas foram incorporadas numa mistura de agar 1,5% e sacarose 10% preparadas em PBS 0,1 M (pH 7,4). Após a solidificação do ágar com as larvas incrustadas foram cortados blocos que foram crioprotegidos por uma solução de sacarose 30% (p/v) preparada em tampão PBS 0,1 M (pH 7,4) durante 24 h. Os blocos de agar contendo as larvas crioprotegidas foram congelados em um criostato (Leica CM 1850) e cortados a -28ºC em seções transversais de 10 µm de espessura. Estes cortes foram coletados em lâminas que foram coradas com hematoxilina-eosina para observar possíveis alterações histológicas. Após o tingimento os núcleos apresentaram uma coloração violeta e o citoplasma uma cor rosada (Figura 12).

Para cada larva, foram obtidos cerca de trinta cortes que foram fotografados a uma ampliação de 100x usando um microscópio (Leica) acoplado a uma câmera digital (OptikamB3) (Prieto et al., 2014).

Tabela 2. Concentrações subletais de PCA, DFB e suas misturas utilizadas nos testes de morfologia e histologia com as larvas 144 hpf de zebrafish.

Compostos/Misturas	CL50-96h/10 (mg/L)
PCA	1.35
DFB	8.12
75% PCA	1.26
50% PCA	1.58
25% PCA	3.3

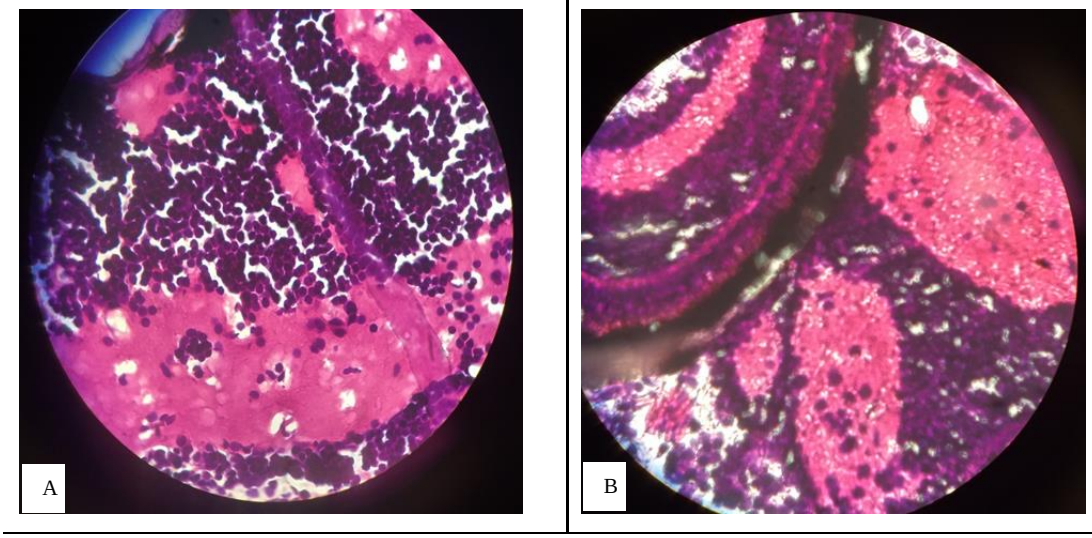

Fig. 12. Seções histológicas da larva de zebrafish com 144 hpf, coradas com eosina-hematoxilina, observadas em microcópio com 40 x de aumento. (A) Fígado e intestino (B) Parte dos olhos, fígado e cérebro.

10. Análise dos resultados

Todos os resultados foram expressos como média (±dp) de, no mínimo, três experimentos realizados em duplicata. Os resultados foram submetidos à análise estatística com o software GraphPad versão 3.05 (GraphPad Software, Inc., San Diego Califórnia USA), utilizando-se de testes de análise de variância (ANOVA) e o teste de Dunnet para comparações de médias em relação ao controle. Valores de *p* iguais ou menores que 0,05 foram considerados estatisticamente significativos.

As concentrações letais (CL50-96h) foram calculadas utilizando a análise de probito (software Statgraphics Plus v. 5.1) e foram consideradas estatisticamente diferentes quando não houve sobreposição dos intervalos de confiança de 95%.

CAPÍTULO I

ESTUDO COM TILÁPIAS *(Oreochromis niloticus)*: EXPOSIÇÃO AGUDA E ANÁLISE DE BIOMARCADORES ENZIMÁTICOS DO FÍGADO

Artigo aceito para publicação: Dantzger, D.D. et al.

Mixtures of diflubenzuron and p-chloroaniline changes the activities of enzymes biomarkers on tilapia fish *(Oreochromis niloticus)* in the presence and absence of soil. Ecotoxicology and Environmental Safety.

ABSTRACT

The insecticide Diflubenzuron (DFB) used by many fish farming when metabolized or degraded produces the extremely toxic compound p-chloroaniline (PCA). Once in the aquatic environment, these compounds can form mixtures and their bioavailability depends on factors such as the presence of soil. The toxic effects of the isolated compounds and their mixtures in the proportions: 75%, 50%, and 25% of PCA were analyzed in tilapia (*Oreochromis niloticus*) in the presence and absence of soil after 96h. The enzymes catalase (CAT), acid (AcP) and alkaline (AlP) phosphatases and alanine (ALT) and aspartate (AST) aminotransferases of the liver of the tilapia were used as biomarkers. DFB and the mixture containing 75% of this compound did not present high toxicity to fish, however, 25 mg/L of PCA alone and 15 mg/L of the mixture with 75% of this compound promoted 50% mortality of tilapia. In the presence of soil, these toxicity values decreased to 37 and 25 mg/L, respectively. Independent of the presence of soil, a synergistic effect was observed when the proportion of PCA was 75% and to the mixture, with 25% PCA was observed the antagonistic effect. Different concentrations of the compounds and their mixtures induced CAT activity independently of the presence of soil. Additionally, increases in phosphatases and transaminases activities were observed. In some cases, the enzymes also had their activities decreased and the dose-dependence effects were not observed. This research showed that the presence of soil influenced the toxicity of the compounds but not altered interaction type among them. Diflubenzuron, p-chloroaniline, and mixtures thereof caused disorders in enzymes important for the health of tilapia (*Oreochromis niloticus).*

1. INTRODUCTION

Diflubenzuron (DFB), a benzoylurea that inhibits the formation of the chitinous exoskeleton, was developed to be used in agriculture in pest control (Branson et al., 2000). Another application of this insecticide was found to control parasites in fish farming, such as *Lernaea cyprinacea* and *Dolops carvalhoi*, with no toxicity for the fish (Eisler, 1992). The effects of DFB in many fish species have been determined and the median lethal concentration that caused 50% mortality (LC50-96h) was calculated to be higher than 50 mg/L (S. a Fischer and Hall, 1992). The LC50-96h of this insecticide for the tilapia fish *(Oreochromis niloticus)* was above 100 mg/L (Jonsson et al., 2015). However, some studies point that such concentrations of DFB can be lethal for zooplankton, mainly those who present chitinous exoskeleton, as freshwater invertebrates, marine and estuarine crustaceans (Henrique et al., 2005). Thus, populations of cladoceran and copepods can be completely eliminated for extended periods of exposure to DFB concentrations above 7 μg/L (S. a Fischer and Hall, 1992). Furthermore, diflubenzuron, when metabolized or degraded, can generate p-chloroaniline (PCA) a highly toxic chemical compound to fish and human (Radomski, 1979; Kataoka, 1996). LC50-96h values of this compound were 2.4, 12 and 14 mg/L to fish Bluegill, Rainbow trout, and Fathead minnow, respectively (Julin and Sanders, 1978). PCA is regarded as a procarcinogen and is known to induce splenic and bladder as well as liver tumors in mammals (Chhabra et al., 1991).

Scenario worrisome and complex is observed where the use of DFB not only in agriculture but also in fish farming generates effluents that can contain this compound and its PCA metabolite compromising the quality of the water body and human health.

Since DFB and PCA remain together in the water environment, it is of interest to know the toxic effects of their joint action in the organisms (Nelson and Kursar, 1999). The bioavailability of DFB and PCA in the water bodies directly depends on the interactions with the constituents of the environment, such as the soil (Ole B. Samuelsen, 2016). Diflubenzuron was found adsorbed in the soil of fish farms for many months, presenting a half-life of 100 days (Samuelsen et al., 2015).

In this context, the use of enzyme activities analyses as biochemical evaluations constitutes an adequate tool of low cost and of a simple application for measuring water toxicity. Alterations in biochemical or molecular levels are normally the first detectable and measurable responses to environmental changes (Huggett et al., 1992).

Fish, important representative organisms in the aquatic food chain, are regularly used as a test in ecotoxicological studies (Di Giulio and Hinton, 2008). In this work, the test organism used was the tilapia fish *(Oreochromis niloticus)*, widely found in fresh water and cultivated and consumed in many countries (Meurer et al., 2003).

The evaluation of enzymes extracted from organs, a mainly fish liver became highly relevant in studies of biomonitoring due to its countless vital functions such as biotransformation and contaminants excretion (Begum, 2005).

Effects of DFB and PCA on enzymatic systems in aquatic organisms are only scarcely discussed in the literature. This lack of study can also be extended to the joint action of the compounds and mainly the toxic effects in the presence of soil. Thus, the objective of this study was to analyze the activity of the enzymes catalase (CAT), phosphatases (acid (AcP) and alkaline (AlP)) and transaminases (alanine (ALT) and aspartate (AST) aminotransferases) of the liver of tilapia *(Oreochromis niloticus)* exposed to different concentrations of the DFB, PCA, and mixtures thereof in the presence and absence of soil.

2. MATERIALS AND METHODS

2.1 Chemicals

Diflubenzuron (DFB), obtained from a commercial formulation (Dimilin®, Chemtura Industria Quimica Ltda, Brazil), contained 25% of the active ingredient and 75% of inert excipients, according to the label information. p-chloroaniline (PCA) technical grade (purity > 99%) was obtained from Sigma-Aldrich.

DFB and PCA were individually tested and in mixtures with the following proportions: 75%, 50% and 25% PCA. For instance, 100 mg/L of mixture with 75%, 50% and 25% PCA contains 75 mg/L PCA and 25 mg/L DFB; 50 mg/L PCA and 50 mg/L DFB; 25 mg/L PCA and 75 mg/L DFB, respectively.

The test solutions were prepared with dechlorinated water.

Analysis of compounds concentrations and the renewal of the test solutions were not performed in the assays due to the stability of the compounds in the physicochemical conditions and the durability of the tests (Zaidi et al., 2013).

2.2 Artificial Soil

The artificial soil used on the tests was prepared by a mixture of 70% autoclaved sand supplied by Institute of Technological Research São Paulo- SP- Brazil, 20% kaolin (CAS Number: 1332-58-7) obtained from Sigma-Aldrich and 10% peat commercial autoclaved (Mineração Darcy, São Simão, SP, Brazil). The soil was prepared according to OECD 207 protocol (OECD, 1984).

2.3 Test organisms

Tilapia fish (*Oreochromis niloticus*) were obtained from a local supplier (Piscicultura Polettini, Mogi Mirim, São Paulo, Brazil) and acclimatized in the laboratory for one month in plastic tanks with 180 L of dechlorinated water and at the temperature of 28 ± 2 °C. The physical and chemical parameters of the water were monitored and remained constant: pH 7.4 ± 0.4; dissolved oxygen 8 ± 2 mg O_2/L; conductivity 160 ± 5 µS/cm and hardness 50 ± 2 mg $CaCO_3$/L.

The animals were exposed under natural light-dark cycle and fed with commercial feed TetraMin Plus (Tetra Holding US Inc.).

All procedures used in the experiments with the tilapia fish (*Oreochromis niloticus*) were approved by the Ethics Committee for the Use of Animals of the State University of Campinas (CEUA/UNICAMP) under the registration Nº 3641-1 (Law Nº 11794/2008).

2.4 Assessment of acute toxicity

After the acclimation period, 10 juvenile tilapia (*Oreochromis niloticus*) with length and average weight of 3 cm and 6 g, respectively, were transferred to glass aquarium containing 10 L of the test solution (5 fish per aquarium and two replicas per concentration) and this system remained static under constant aeration and controlled temperature (28 ± 2°C). This test was performed in the presence and absence of soil. For the tests in the presence of soil, fish were transferred to glass aquarium containing 900 g of artificial soil and 10 L of test solution under conditions above mentioned.

The concentrations evaluated of diflubenzuron, p-chloroaniline and their mixtures with the proportions of 75%, 50% and 25% PCA were: 0.0; 0.1; 1.0; 10.0 and 100.0 mg/L (OECD, 1992). Namely, the concentration 0.1 mg/L of the mixture with 75% PCA contains 0.075 mg/L PCA and 0.025 mg/L DFB; the mixture with 50% PCA contains 0.05 mg/L PCA and 0.05 mg/L DFB; the mixture with 25% PCA contains 0.025 mg/L PCA and 0.075 mg/L DFB

The total period of exposure the fish to concentrations of compounds and mixtures thereof were 96 hours, during which the organisms were not fed. At the end of the exposure period, the number of dead individuals was registered in order to determine the lethal concentrations that affect 50% of the population (LC50-96h). LC50-96h values were calculated using probit analysis (Statgraphics Plus v. 5.1 software) and were considered to be statistically different when there was no overlap of the 95% confidence intervals.

2.5 Samples preparations and biochemical analyses

For biochemical analyses, tilapias were exposed to sublethal concentrations of the compounds and mixtures thereof (75%, 50% and 25% PCA). These concentrations were calculated from the LC 50-96h values, obtained on acute assessment, divided by 10, 50 and 100. For example, considering the LC50-96h value of 100 mg/L for the mixture with 50% of each compound, the sublethal concentrations were 10, 5, and 1 mg/L. To calculate the proportions of the same mixture above mentioned, in the case of 10 mg/L, it was used 5 mg/L of each compound

For the tests, 10 adult tilapia (*Oreochromis niloticus*) (5 fish per aquarium and two replicas per concentration) were transferred to glass aquaria containing 10 L of the solution test and this system remained static under constant aeration and controlled temperature (28 ± 2°C) (OECD 1992). The total period of exposure of fish to sublethal concentrations of DFB, PCA, and mixtures thereof was 96 hours, during which fish were not fed. This test was performed in the presence and absence of soil. For the tests in the presence of soil, fish were transferred to glass aquarium containing 900 g of artificial soil and 10 L of test solution under constant aeration and controlled temperature (28 ± 2°C).

After 96 h, fish were collected, anesthetized with benzocaine (0.1 g/L) and sacrificed by spinal cord section, accordance with the principles of the Ethics Committee for the Use of Animals of the State University of Campinas (CEUA/UNICAMP) to avoid the suffering of organisms. Thereafter the liver of these fish was removed and divided into equal portions, weighed and homogenized in the appropriate buffer for each enzyme at a ratio of 1:4 (weight/volume). This homogenate was centrifuged at 10.000xg, for 20 min, at 4°C and the supernatant was collected. The supernatant was stored at - 80°C and after used for the biochemical analyses, comprising the specific activities of catalase (CAT), alkaline (AlP), and acid phosphatase (AcP), alanine (ALT) and aspartate (AST) aminotransferases as well as the protein concentration.

2.5.1 Acid (AcP) and Alkaline (AlP) Phosphatases

AcP and AlP activities were determined through the p-nitrophenol (pNP) formed, using p-nitrophenyl phosphate (pNPP) as a substrate for enzymes, according to the method previously described (Jonsson and Aoyama, 2007). For AcP activity, 10 µL of supernatant containing the enzyme was added to 15 µl of 100 mM acetate buffer (pH 5) and 125 µl of 10 mM p-nitrophenyl phosphate (pNPP) solution. AlP activity was measured like AcP, except for addition 15 µL of 250 mM glycine buffer (pH 9.4) and 10 µL of 20 mM $MgCl_2$ as a cofactor. The mixtures were incubated for 40 min at 37 °C, after, the reaction was stopped by adding 50 µL of 1 M NaOH, followed by measuring the absorbance at 405 nm, due to the formation of pNP with yellow-colored (molar extinction coefficient = 18.000 $M^{-1}cm^{-1}$) (Jonsson and Aoyama, 2007). The specific activity of AcP and AlP was expressed in nmoles $pNP.min^{-1}.mg\ protein^{-1}$. The analyses were performed in triplicate and employed a microplate spectrophotometer EON BioTek.

2.5.2 Catalase (CAT)

The specific activity of CAT (nmoles of $H_2O_2.min^{-1}mg\ protein^{-1}$) was determined according to the method described by Aebi (Aebi, 1984). For this, 50 µL of the supernatant containing the enzyme was added to 250 µL of hydrogen peroxide 0.03M (substrate) prepared in 50 mM phosphate buffer at pH7. Then, the absorbance at 240 nm was monitored for 1 min in microplate spectrophotometer EON BioTek.

2.5.3 Transaminases (AST/ALT)

The activities of aspartate (AST) and alanine (ALT) transaminases in the supernatant were measured using commercial kits Bioliquid, obtained from Laborclin (São Paulo, Brazil). The enzyme AST produced oxaloacetate that was reduced to malate by malate dehydrogenase in the presence of NADH. The decrease of NADH, measured at 340 nm in microplate spectrophotometer EON BioTek, was proportional to the activity of AST. Pyruvate produced by ALT was reduced to lactate by the enzyme lactate dehydrogenase, in the presence of NADH, which decrease was proportional to the activity of ALT. Both the dehydrogenases are present with the substrates in the commercial kit. According to the kit, the enzyme activities were expressed as U/mL.

2.6 Concentration Protein

For the determination of the specific activity of the enzymes, the protein of supernatant was quantified by the Lowry method (Lowry et al. 1951) at 560 nm in microplate spectrophotometer EON BioTek, using bovine serum albumin as standard.

2.7 Statistical analysis

Lethal concentrations (LC50-96h) were calculated using probit analysis (Statgraphics Plus v. 5.1 software) and were considered to be statistically different when there was no overlap of the 95% confidence intervals.

For each type of exposure (with and without soil), one-way ANOVA was used to analyze the biochemical data. Comparison of the groups treated and control employed the Dunnett test and differences were considered significant at $P < 0.05$ or $P < 0.01$. GraphPad Prism v. 5.1 software was used and data were presented as mean ± SD.

3. RESULTS

3.1 Acute toxicity test

Tilapia *(Oreochromis niloticus)* were exposed to concentrations ranging from 0 to 100 mg/L of DFB, PCA and binary mixtures of these compounds containing 75, 50, and 25% of PCA. In the acute toxicity test, DFB and the mixture containing 25% of PCA this compound did not present toxicity to fish, with an LC50-96h value higher than 100 mg/L, both in the presence and in the absence of soil. However, only 25 mg/L of the metabolite PCA alone and 15 mg/L of the mixture with 75% of this compound promoted 50% mortality of tilapia without soil. In the presence of soil, these toxicities values decreased and the LC50-96h obtained for PCA alone and in the mixture, with 75% PCA were 37 and 25 mg/L, respectively. LC50-96h values of 40 mg/L and 45 mg/L were obtained for the mixture 50% of PCA, in the absence and in the presence of soil, respectively.

Tilapia *(Oreochromis niloticus)* percentage of mortality in the acute toxicity bioassays can be observed in Fig.1.

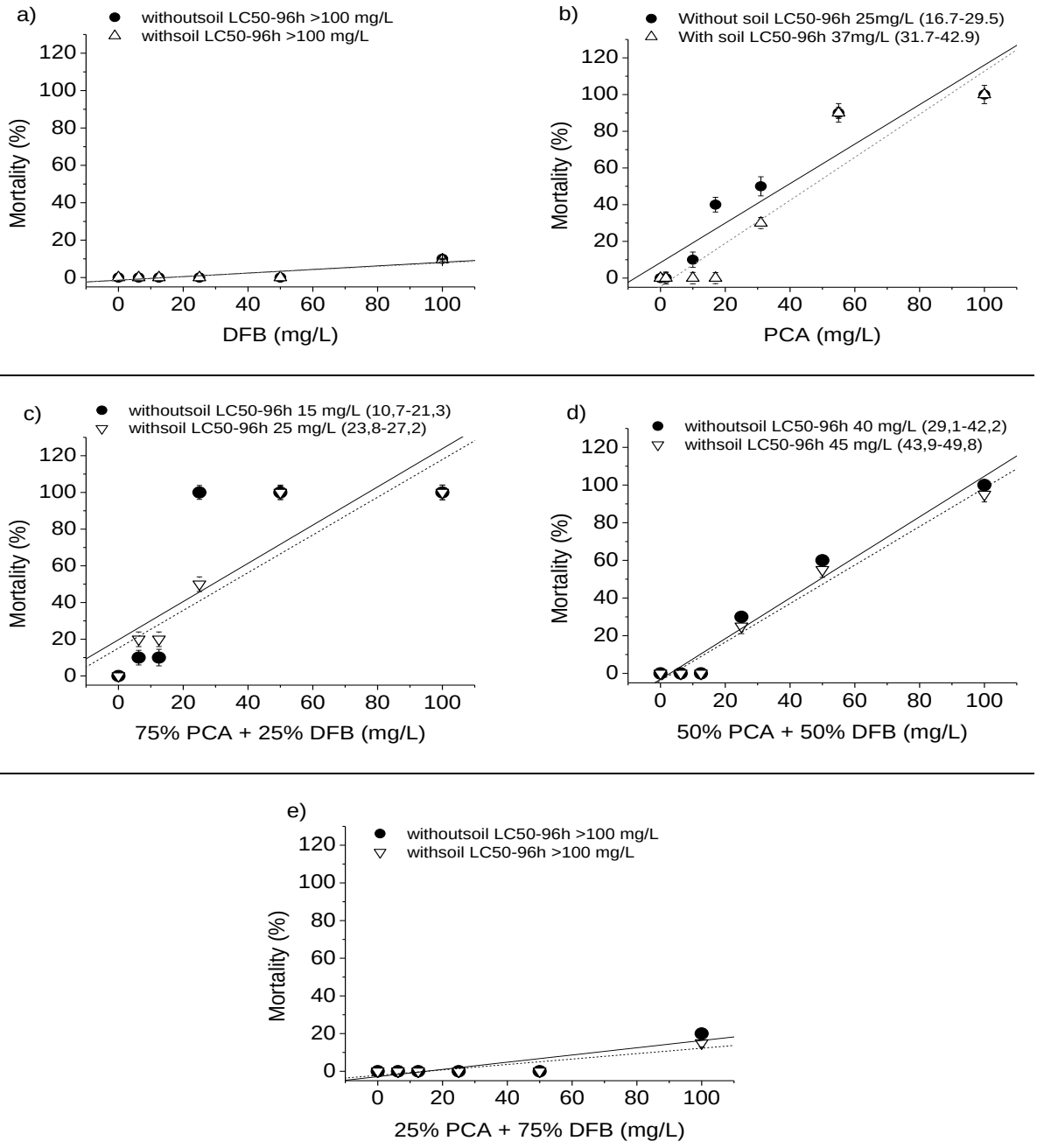

Fig. 1. Percentage of mortality (mean value ± standard error) of tilapia *(Oreochromis niloticus)* (n=10) exposed to different concentrations of DFB, PCA, and their mixtures for 96 h.

3.2 Enzymatic Analysis

3.2.1 Enzyme activities without soil

The isolated compounds and their mixtures caused an increase in the activity of catalase in the absence of soil, with the exception of 75% PCA (Fig. 2). At the same conditions, PCA alone promoted a decrease in the CAT activity (Fig.2). The concentrations of 1, 2 and 10 mg/L of the mixture containing 25% PCA caused significant increases of 254% ($P < 0.05$),

150% ($P < 0.01$), and 282% ($P < 0.05$), respectively, in the CAT activity (Fig. 2). A similar effect was also observed for DFB isolated (Fig. 2).

The acid phosphatase (AcP) activities increased by exposure of tilapia *(Oreochromis niloticus)* to PCA and mixtures thereof. The most significant increases ($P < 0.01$) (80%) were obtained with 10 mg/L of the mixture with 25% PCA (Fig. 3) and 1.5 mg/L ($P < 0.01$) of 75% PCA (Fig. 3). No significant change was observed in the AcP activity when exposed to DFB isolated (Fig. 3).

Increases of 42% ($P < 0.01$), 54% ($P < 0.01$), and 38% ($P < 0.05$) in the alkaline phosphatase (AlP) activities were observed for 0.15, 0.3 and 1.5 mg/L of mixtures of 75% PCA (Fig. 3). At these conditions, the mixture with 25% PCA decreased 49%, 55% and 43% ($P < 0.01$) the AlP activities (Fig. 3).

The activity of the transaminase ALT increased in the presence of the compounds separately and in the mixture of 50% PCA ($P < 0.05$). However, in the presence of mixtures containing 75% and 25% PCA the activity of this enzyme decreased ($P < 0.01$) (Fig. 4). All the activities of the transaminase AST decreased excepting by exposure to the mixture of 50% of each compound ($P < 0.01$) (Fig. 4).

3.2.2 Enzyme activities with soil

In the presence of soil, the CAT activities decreased in contrast to observed for this same enzyme in the absence of the soil (Fig. 2). However, in the presence of soil, CAT activity increased ($P < 0.01$) by exposure to PCA isolated and to 50% of the mixture with PCA (Fig. 2).

The acid phosphatase activity increased ($P < 0.01$) in the presence of the mixture with 25% PCA (Fig. 3). On the other hand, decreases of 48%, 43%, and 17% ($P < 0.01$) of its activity were observed for 1, 2, and 10 mg/L of DFB alone (Fig. 3). At a lesser extent, it was also observed a decrease ($P < 0.01$) in the acid phosphatase activity of the mixture 50% of PCA (Fig. 3).

Exposure of tilapia *(Oreochromis niloticus)* to PCA isolated and in a mixture of 50% this compound promoted increases ($P < 0.01$) of 38% and 65%, respectively, in the activities of alkaline phosphatase (Fig. 3).

In the presence of soil, the transaminase ALT activity increased for the three mixtures of DFB and PCA (Fig. 4). On the other hand, the AST activity increased ($P < 0.01$) in the presence of the PCA and DFB, separately (Fig. 4).

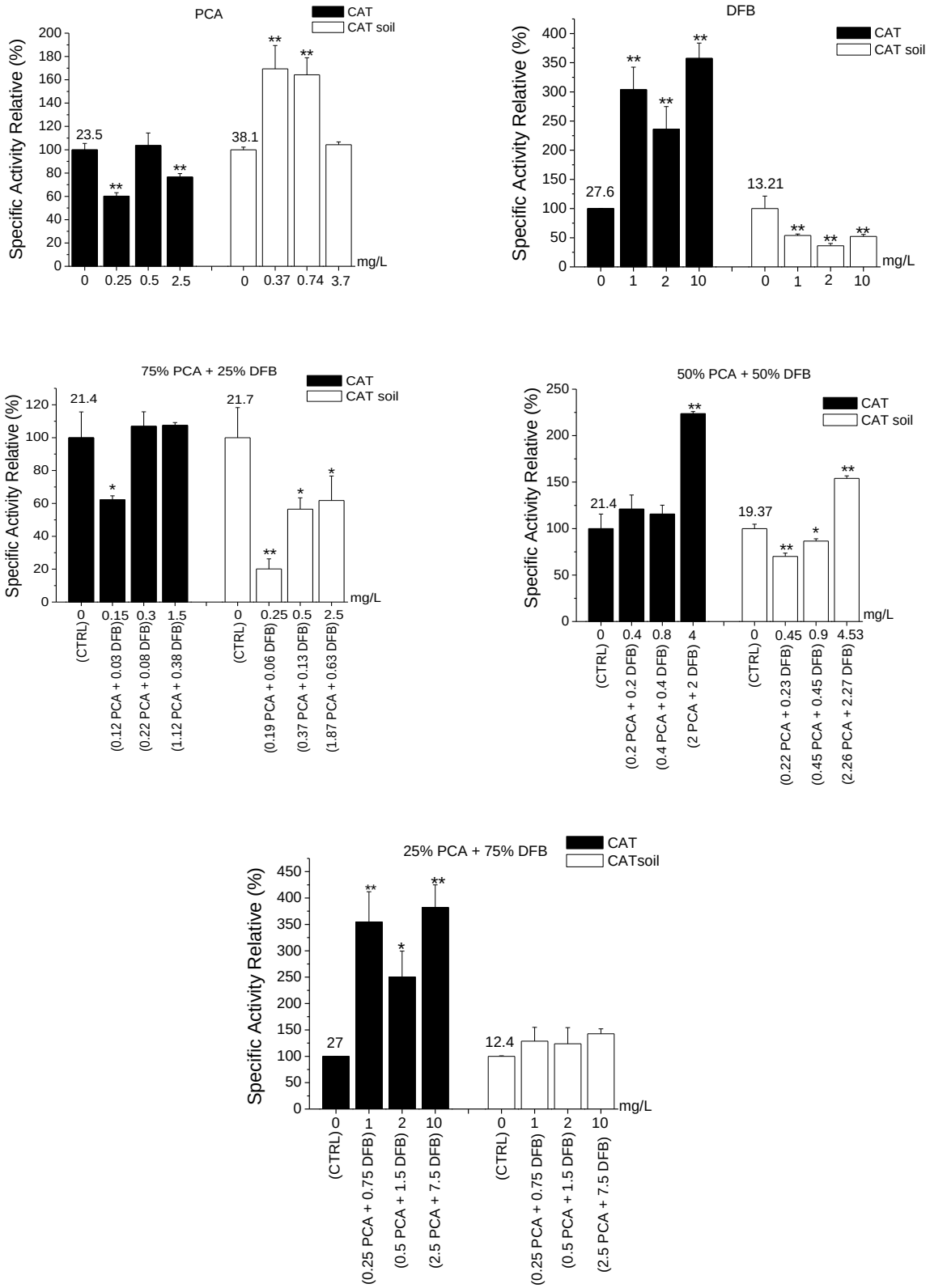

Fig. 2. Effect PCA, DFB, and mixtures thereof on catalase (CAT) activities of tilapia liver (n =10) after 96h of exposition, in presence and absence soil. The numbers that appear on the control bars (zero concentration) referring to 100% activity correspond to nmol of H_2O_2 decomposed per min per mg of protein. *Significantly different from control at P<0.05 and ** P<0.01. Data are expressed as mean (±standard deviation).

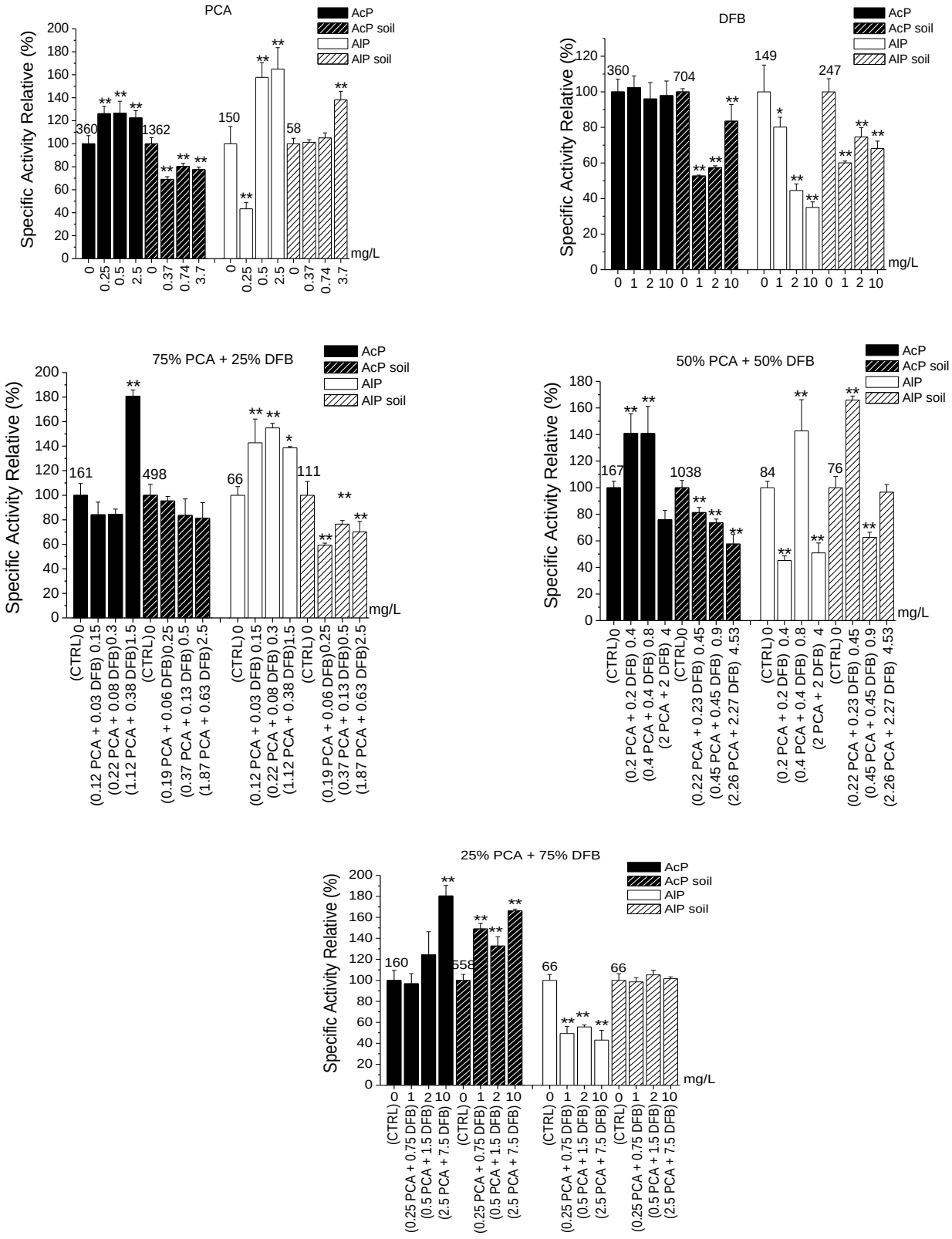

Fig. 3. Effect PCA, DFB, and mixtures thereof on Acid Phosphatase (AcP) and Alkaline Phosphatase (AlP) activities of tilapia liver (n = 10) after 96h of exposition, in presence and absence soil. The numbers that appear on the control bars (zero concentration) referring to 100% activity correspond to nmol of p-nitrophenol produced

per min per mg of protein. *Significantly different from control at P<0.05 and ** P<0.01. Data are expressed as mean (±standard deviation)

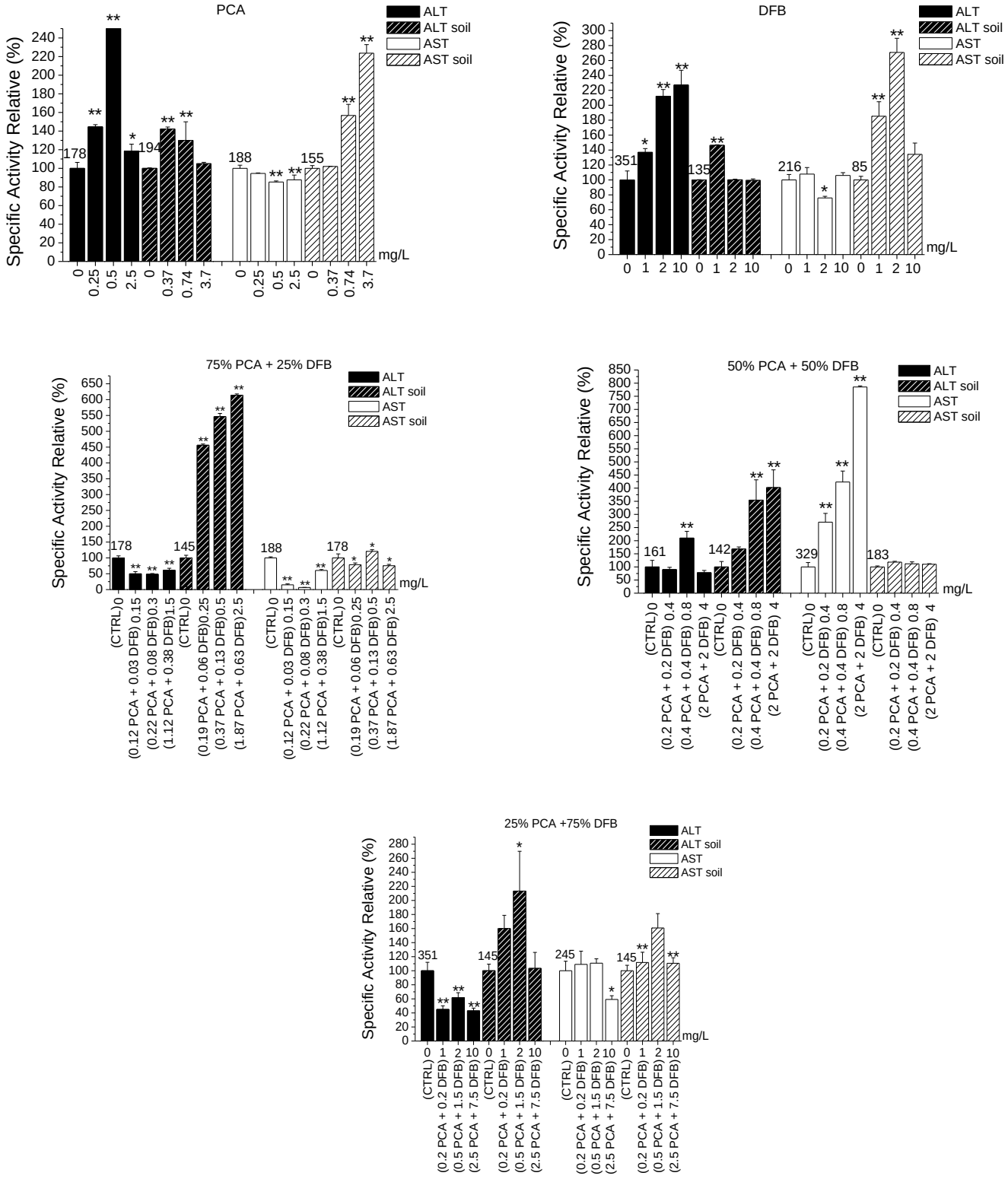

Fig.4. Effect PCA, DFB, and mixtures thereof on aspartate (AST) and alanine (ALT) transaminases activities of tilapia liver (n = 10) after 96h of exposition, in presence and absence soil. The numbers placed on the control bars

(zero concentration) referring to 100% activity correspond to U/mL of NADH consumed. *Significantly different from control at P<0.05 and ** P<0.01. Data are expressed as mean (±standard deviation).

4. DISCUSSION

In the acute toxicity test, DFB isolated and in the mixture with 25% PCA did not show significant toxicity on the tilapia fish (*Oreochromis niloticus)* with LC50-96h values higher than 100 mg/L, both in the absence and in the presence of soil. The absence of toxicity of DFB for fish was also reported by other authors. LC50-96h values of 100, 240 and 370 mg/L were obtained for tilapia (Jonsson et al., 2015), Rainbow trout and Channel catfish (Fischer and Hall, 1992), respectively. However, Lee and Scott (1989) observed DFB toxicity for *Fundulus heteroclitus* fish, with an LC50-96h value of 33 mg/L.

These doses can be lethal to zooplankton populations, mainly for those with a chitinous exoskeleton (Nebeker et al., 1983). Some authors do not recommend the use of DFB in fish farming due to the risk for these organisms (Fischer and Hall, 1992). Mabilia et al. (2006) evaluated the DFB toxicity in effluents of fish farming and obtained medium effective concentration (EC50-48h) values of 0.18, 0.51, 0.05, 0.039 μg/L for the micro crustaceans *Daphnia similis*, *Ceriodaphnia dubia*, *Ceriodaphnia silvestrii* and *Simocephalus serrulatus*, respectively. Moreover, we have observed that 25 mg/L of PCA isolated and 15 mg/L of the mixture with 75% PCA were able to kill 50% of fish after 96h, in the absence of soil. In the presence of soil, LC50-96h of 37 mg/L and 25 mg/L were obtained for PCA isolated and in the mixture with 75% PCA, respectively. These results suggest a decrease in the PCA toxicity due to the presence of soil. PCA showed to be more toxic than DFB for the fish Bluegill, Rainbow trout, and Fathead minnow, with LC50-96h values of 2.4, 14 and 12 mg/L, respectively (Julin and Sanders, 1978).

In the absence of soil, the LC50-96h value obtained for the mixtures of DFB and PCA containing 50% of each compound was 40 mg/L; in the presence of soil, the toxicity slightly decreased, with an LC50-96h value of 45 mg/L. These data displayed that DFB alone in the aquatic environment does not show toxicity for fish, however, in the presence of its metabolite PCA, synergism and antagonism effect can be observed. A synergistic effect was observed when the proportion of PCA was 75% and the toxic effect of the compounds on tilapias was more severe. To mixture with 25%, PCA was observed the antagonistic effect.

This information is relevant since in the aquatic environment DFB and PCA are found in mixtures, which combined effect was only scarcely discussed in the literature.

Our results showed that the presence of soil contributed to diminishing the toxicity of PCA individually or in combination with DFB but did not change the type of interaction that occurred between the compounds in the mixtures. The soil can be considered an important factor in the evaluation of the toxicity of PCA and DFB in the aquatic environment. In the aquatic environment, the compounds can associate to particles of the soil diminishing its availability to the organisms (Haya et al., 2005). Therefore, the presence of soil is essential in tests to define the maximum permissible limits of xenobiotics in the aquatic compartment.

Low mobility and low leachability of DFB in soil are responsible for its high degree of adsorption in organic matter (Schaefer et al., 1979; Eisler, 2000). The persistence of DFB and PCA in the soil depends on factors such as particle size, temperature, pH and soil composition (Chapman et al., 1985). In this work, the conditions physical-chemicals of assays (pH=7.4, temperature 28°C), probably favored the adsorption of the compounds to soil particles. Due to its low solubility, DFB may have a preference to the soil when present in the aquatic environment (Sundaram et al., 1991). Furthermore, DFB is stable to hydrolysis at pH values of 5 to 7, with a reported half-life hydrolysis of 32 days, at pH 9 (Eisler, 1992). Moreover, the persistence of DFB in soil is microbe-dependent; its half-life under field conditions ranges from 7 to about 19 days (Nigg et al., 1986).

Under aerobic conditions, PCA may covalently bind to soil particles, particularly in the presence of high amounts of organic material and under low pH levels (Gawlik et al., 1998). However, under unfavorable conditions for abiotic and biotic degradations, it can be expected leaching of PCA from soil with a low organic matter content and elevated pH levels into groundwater (Worobey and Webster, 1982).

The continuous exposition at low concentration of pollutants and complex mixtures has demanded the development of more rapid and sensible methods when compared with analysis of acute toxicity (Segner and Braunbeck, 1990).

Alterations at the biochemical or molecular levels, in general, are the first detectable and quantifiable responses by changing the environment. Variation of biochemical systems is frequently more sensible indicators than the great biological organization levels, such as cells, organisms, and population (Hinton et al., 2005). Due to these characteristics, the biochemical biomarkers are pointed out as systems of alert signal in the evaluation of environmental health (Diamantino et al., 2000)

In order to verify the contamination of water body by chemicals, such as DFB and PCA, the utilization of biochemical indicators can be economically more advantageous when

compared with the implementation of protocols of analyses using high-cost methods and that demand higher time of analysis (Arias et al., 2007).

Enzymes are the major substances involved in the metabolism and detoxification of foreign compounds in living organisms (Ling et al., 2011). Some pollutant agents can concentrate in different tissues and promote induction or inhibition of enzymatic systems responsible for crucial cellular processes, such as those of phosphatases, transaminases, and catalase (Jonsson and Aoyama, 2007). Thus, enzymes have been used as biomarkers of environmental pollution in ecotoxicology studies (Moore et al., 2004).

The oxidative damage induced by chemicals in aquatic ecosystems can be assessed through the measurements of antioxidant enzyme activities in fish (Slaninova et al., 2009). Catalase is an antioxidant enzyme that protects organisms against hydrogen peroxide (H_2O_2) generated in many situations of stress (Oruç and Uner, 2000). In this, sublethal concentrations of DFB, PCA, and their mixtures induced a significant increase of CAT activities after exposure of tilapia for 96 h (Fig. 2). It is possible that the increased activities of this enzyme contribute to the elimination of reactive oxidant species (ROS) in the organism induced by compounds exposure. Similar results were also found for *P. lineatus* fish, after 96 h exposure to DFB, where the induction of CAT was probably due to the increased production of hydrogen peroxide during the metabolism of the insecticide (Pereira Maduenho and Martinez, 2008a).

The activity of catalase decreased in some lethal concentrations of DFB, PCA and of the mixture. The decreased activity of CAT in the liver of treated fish may indicate the highly reduced capacity to scavenge hydrogen peroxide produced in this tissue, with an increase in ROS and oxidative stress in response to acute intoxication with compounds. Oxidative stress occurs when ROS production overcome the endogenous protection promoted by specific degradative enzymes, antioxidant vitamins, and other radical scavengers, causing damage to cellular constituents (Hamed et al., 2016).

Inhibition of CAT was observed in the liver of Zebrafish at high concentrations of Trichlorfon (Coelho et al., 2011). The exposure of Goldfish to the herbicide Sencor decreased by 31-34% the CAT activity (Husak et al., 2014).

Phosphatases are enzymes that carry out important metabolic functions such as the decomposition of organic phosphates to free inorganic phosphates and are also involved in intracellular signaling process (Aoyama et al., 2003). Acid phosphatase (AcP) is considered a lysosomal hydrolase biomarker since its leakage to intra and extracellular fluids occurs in a situation of cellular stress. Alkaline phosphatase (AlP) is a lysosomal and plasma membrane biomarker due to its adaptive response to the cytotoxic and genotoxic effects of xenobiotics and

its pivotal role in the transport of metabolites across the membranes (Suresh et al., 1993). In this work, the AcP and AlP activities have increased and decreased by exposure to the compounds in the presence and absence of soil (Fig. 3). The increased phosphatases activities can be attributed to the disruption of the cell membrane and lysosomes inferring possible liver damage like an obstruction in the intro and extrahepatic biliary system (Lohner et al., 2001). The decrease in phosphatase activities may be due to histopathological changes such as necrosis (Rs and Shaikh, 2013; Mir et al., 2016). On the other hand, the reduction observed in the phosphatase activities can be related to the toxic effects of DFB and PCA on tilapia liver. An increase in the alkaline phosphatase activity was observed in tilapia when exposed to cipermethrin (Fırat et al., 2011). Mir et al. (2016) observed a decrease in the liver and kidney acid and alkaline phosphatase activities of the freshwater fish *Labeo rohita* by exposition to heavy metals. Although several works have shown differential effects on the activities of phosphatases, in the literature the data on these enzymes in aquatic organisms exposed to DFB and PCA are only scarcely mentioned.

The enzymatic tests of hepatic function constitute a form to study the liver and its possible functional alterations. The transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes involved in the metabolism of amino acids, and alterations in their activities can allow the identification of tissue damage in organs such as liver and kidney (Begum, 2005). DFB and PCA, separately, and in the mixtures increased the activities of the transaminases for almost all the concentrations evaluated to tilapia (Fig. 4).

2 mg/L DFB and 0.5-2.5mg/L-PCA, separately, decreased the AST activities. Das et al. (2004) assigned the reduced activities of AST and ALT to an inactive transamination and oxidative deamination or destruction of the tissues. Differential effects on the activities of these enzymes have been detected by other authors. Inhibitions in the activities of AST and ALT were observed in various organs of the African catfish *Clarias gariepinus* after 10 days of exposure to sublethal levels of the pesticide cypermethrin (Gabriel et al., 2012). Whereas an increase of AST and ALT activities were observed in *O. niloticus* fish when exposed to 1.46 µg/L of the pesticide deltamethrin (Sapana Devi and Gupta, 2014).

In contrast to the acute toxicity study, the presence of soil did not significantly affect the enzymatic assays. The enzymes CAT, AcP, AlP, AST, and ALT had their activities altered, even though dose-dependent effects were not generally observed, in the presence and in the absence of soil. Olawale et al. (2005) did not observe a consistent dose-response relationship in the enzymatic activities; low concentrations of a xenobiotic might inhibit an enzyme activity, while higher concentrations might have no effect.

Our results revealed that sublethal concentrations of DFB and PCA caused severe metabolic disorders in the tilapia fish. Once this fish is intensively cultivated and consumed in several countries, these results are worrying. Several studies have been performed about different forms to reduce pesticides toxicity in tilapia health, as the use of natural products in its diet. Spirulina (microalgae) dietary supplementation, rich in antioxidant active constituent, reduced toxic effects caused by synthetic pyrethroid insecticide Deltamethrin in tilapias (*Oreochromis niloticus)* (Abdelkhalek et al., 2015). Allicin found and extracted of garlic clove extracts enhanced altered serum biochemical parameters by deltamethrin in tilapia (*Oreochromis niloticus)* for 28 days (Abdel-Daim et al., 2015).

These natural products could be used in fish culture tanks to minimize the effects of DFB on tilapia and, consequently, protect the health of people who consume their protein. However, when the DFB and its metabolite reach the aquatic environment other care must be taken because the health of all ecosystem can be affected. A study with aquatic organisms of different trophic levels including tilapia showed that the toxicity of DFB decreases after the use of gravel and expanded clay in aquarium water filters (Jonsson et al., 2015).

Finally, our results reinforce that alteration in the enzymatic activities of CAT, AcP, AlP, AST and ALT in the liver of tilapia fish may be considered sensitive biomarkers in ecotoxicological studies, early predicting contamination of aquatic organisms with diflubenzuron and p-chloroaniline and, ultimately, serving as a tool of eco-monitoring.

5. CONCLUSION

This research showed that the presence of soil influenced the toxicity of the compounds and that diflubenzuron, PCA, and mixtures thereof caused disorders in enzymes important for the health of tilapia. Therefore, these compounds should be used with caution.

This work reinforces the importance to study the joint action of compounds when two or more of them are being considered as an aquatic pollutant and also the presence of soil that can alter the toxicity of these compounds.

The results obtained in this study, fill in part, a gap in the scientific literature regarding the environmental impact of DFB and its metabolite PCA on aquatic organisms. The results are of importance to support both environmental programs of improvements to fish farming areas as to regulatory government agencies. The present research suggests that further work is needed in order to minimize environmental concentrations of DFB and PCA and to test the long-term effects of these compounds.

CAPÍTULO II

ESTUDO COM TILÁPIAS *(Oreochromis niloticus)*: EXPOSIÇÃO AGUDA, ANÁLISE DE BIOMARCADORES ENZIMÁTICOS DAS BRÂNQUIAS E O USO DO MODELO PARA PREDIÇÃO DE TOXICIDADE DAS MISTURAS DE DFB E PCA.

Artigo submetido para publicação: Dantzger, D.D. et al.

Acute exposure to mixtures of diflubenzuron and p-chloroaniline in the presence and absence of soil causes disorders in enzymes of tilapia fish *(Oreochromis niloticus).*

Ecotoxicology.

ABSTRACT

The insecticide Diflubenzuron (DFB) has been used in several fish farmers to control ectoparasites that cause disease in fish and, in the aquatic environment, this compound can be degraded or metabolized, produces the toxic metabolite p-chloroaniline (PCA). Aquatic communities are usually subject to mixtures of these compounds and their bioavailability depends on factors such as the presence of soil. Mixtures of these compounds can suffer interactions (antagonism, addition, and synergism) and some methods allow the study and the classification of such chemical interactions. The toxic effects of the DFB, PCA and their mixtures in the proportions: 75%, 50% and 25% PCA were analyzed in tilapia fish (*Oreochromis niloticus*) in the presence and absence of soil after 96h. The enzymes catalase (CAT), acid (AcP) and alkaline (AlP) phosphatases of the tilapia gills were used as biomarkers. The Index of Additive was calculated which provided the type of interaction between the compounds in the mixtures. The soil decreased the bioavailability of the compounds in water and an antagonistic and synergistic relationship occurred in the mixture with 25% and 75% PCA, respectively. The fish presented oxidative stress and possible damages in the gills with alterations in the activity of the CAT, AcP and AlP enzymes. These products should be used with caution, since that may pose a risk to aquatic biota and human health.

1. INTRODUCTION

Fish are sources of food, income, and livelihoods for countless people worldwide. Fish farming is an economic activity that had a significant growth in the last fifty years. One million tons of fish were produced in 1950, already in 2014, the production was about 73.8 million tons (F A O, 2016). With the goal of increasing development of this activity, a reduction in mortality of fish with diseases caused by ectoparasites is a basic factor. To this end, the use of chemicals such as insecticides has been constantly employed (Boyd and Massaut, 1999; Branson et al., 2000; Pereira Maduenho and Martinez, 2008b; Ole B Samuelsen, 2016). Diflubenzuron (DFB) (Fig. 1) is an insecticide belongs to the benzoyl urea group and has been used as veterinary medicines in several fish farmers to control ectoparasites by inhibitory action of chitin synthesis (Branson et al., 2000; Macken et al., 2015). In fish farming, the DFB apparently does not cause toxic effects to fish, but there are studies that demonstrate numerous physiological and biochemical effects that can affect the health of these organisms (Fischer and Hall, 2008; Pereira Maduenho and Martinez, 2008a; Schaefer et al., 1979; Zaidi et al., 2013). In addition, DFB in the aquatic environment can suffer degradation or metabolization, producing several metabolites, among them, the p-chloroaniline (PCA) (Fig.2) (Olsvik et al. 2013). PCA is widely used as intermediate in production rubber, dyes, agricultural chemicals and pharmaceuticals. However, it is considered a priority pollutant in the environmental risk assessment for being highly toxic to a variety of aquatic organisms, including fish, and is potentially carcinogenic and mutagenic in humans (Könnecker, G. Boehncke, A. Schmidt, 2003; Sihtmäe et al., 2010).

Leachate of agricultural areas, sewage industrial, and effluent from fish farms can contain these compounds that, if reach the aquatic environment, can cause significant changes. Aquatic communities are usually subject to complex contaminant mixtures, and with the presence of soil, but many laboratory experiments are performed with only one chemical compound which does not reflect the reality in the field. (Ensenbach and Nagel, 1995; Pérez et al., 2013). Mixtures of these compounds can suffer interactions (including antagonism, addition, and synergism) and some methods allow the study and the classification of such chemical interactions (Plackett and Hewlett 1948; Marking 1977). Additivity can occur when two compounds act independently on the same target and their effects are additive. Synergism is defined as an interaction among compounds producing a higher effect when compared with the individual effect of each compound. Contrary, antagonist compounds would reduce the effect (Belden et al., 2007; Jonsson and Aoyama, 2007). In addition, these interactions the

presence of soil can interfere in the bioavailability of the compounds (Samuelsen et al., 2015). Diflubenzuron was found in organic material attached of soil in the fish farms, in contact with the benthic community (Macken et al., 2015; Ole B. Samuelsen, 2016). Natural processes (storms) or human (dredging activities) may resuspend DFB adsorbed on the soil, making it more bioavailable to other aquatic animals and may pass into the food chain (Martins et al., 2012; Ole B Samuelsen, 2016; Ole B. Samuelsen, 2016). Fish are incessantly exposed to mixtures of the xenobiotics throughout their life as are animals that have skin and gills highly permeable and thus more vulnerable to pollutants (Bizarro et al., 2016; Di Giulio and Hinton, 2008). The fish tilapias (O*reochromis niloticus)* is species originating in the rivers and African lakes have gained increasing commercial in the world because its creation is easier, less expensive and the quality of their meat is high (F A O, 2016). Toxicity and biochemicals tests have shown that this species is sensitive to a variety compounds so it can be considered as a potential bioindicator (Carvalho et al., 2012; Fırat et al., 2011; Jonsson et al., 2015; Meng et al., 2013). Exposure of these fish to contaminants as DFB and PCA can cause biological changes in these organisms. These changes can be measured and used as biomarkers that enable the rapid assessment of the health of organisms and alert about possible environmental changes (Böttcher and Schroll, 2007; Dosnon-Olette et al., 2010). Fish gills are in direct contact with water, for such reason, changes on enzyme activities of these organs can be assessed as biomarkers of pollution in the aquatic environment (Bizarro et al., 2016; Lang et al., 2006). On this context, this research analyzed changes in enzymes catalase (CAT), acid (AcP) and alkaline (AlP) phosphatases of gills of tilapia exposed to mixtures of DFB and its metabolite PCA. Phosphatases enzymes can be used to indicate the tissue damage of liver and gills, and catalase is considered a sensitive indicator of oxidative stress (Loteste et al., 2013; Thirumavalavan, 2010).

Although these enzymes are widely used as environmental pollution biomarkers not found in the literature studies on the effects of a mixture of DFB and PCA on the activity of these enzymes. Since it is known regarding the use and disposal of these compounds is frequently, is necessary to investigate the possible damage they cause on the aquatic biota and to humans. Therefore, the objective of this research was to analyze the toxic effects of a mixture of the DFB and PCA on tilapia fish (*Oreochromis niloticus)* in presence and absence of soil, using biochemical parameters as indicators of toxicity.

2. MATERIALS AND METHODS

2.1 Test organisms

Adults and juveniles of tilapia fish (*Oreochromis niloticus)*, were obtained from the local fish farming (Aquaculture Poletini, Mogi Mirim, São Paulo, Brazil).

Fish were acclimated in the laboratory, at the Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas (UNICAMP) for one month in 180-liter plastic tanks containing dechlorinated water, under constant aeration and natural light-dark cycle and temperature of 28 ± 2 °C. The physical and chemical parameters of the water were monitored and remained constant during the acclimation period: pH 7.4 ±0.4; dissolved oxygen 8 ± 2 mg O_2/L; conductivity 160 ± 5 µS/cm and hardness 50 ± 2 mg $CaCO_3$/ L.

During acclimation period, the animals were fed with commercial fish food TetraMin Plus (Tetra Holding US Inc.).

All experiments with the fish were approved by the Ethics Committee for the Use of Animals of the State University of Campinas (CEUA/UNICAMP) under the registration Nº 3641-1 (Law Nº 11794/2008).

2.2 Acute toxicity tests

The acute toxicity bioassays were performed using the commercial formulation of Diflubenzuron, Dimilin®, (wettable powder 25% active ingredient, Chemtura, Brazil), and, p-chloroaniline (PCA) technical grade, purity > 99%, obtained from Sigma-Aldrich. Chemicals were tested individually and in mixtures with the proportions: 75%, 50% and 25% of PCA. The test solutions were prepared with carbon-filtered water.

Analysis of compounds concentrations and the renewal of the test solutions were not performed in the assays because the degradation time of PCA and DFB in water is 6 and <7 days at temperature 28°C and pH 7.7, respectively (Ekici et al. 2001; Zaidi et al. 2013).

The test concentrations of diflubenzuron, p-chloroaniline and mixtures thereof evaluated were 0.0 (control group); 0.1; 1.0; 10.0 and 100.0 mg/L (OECD, 1992) . The tests were performed in the presence and absence of artificial soil that was prepared by mixing the sand (70%), kaolin (20%) and peat (10%) (OECD, 1984).

Ten juvenile tilapia (5 fish per aquarium and two replicas per concentration) were exposed to test solutions in glass aquaria of 10 L with continuous aeration, controlled temperature (28 ± 2°C) and 900g of soil, for tests in presence of this, (Medeiros et al., 2013).

This procedure was realized in duplicate and during the test, fish were not fed. The physical and chemical parameters of the water were monitored and remained the same as the acclimation period.

Every day the number of dead individuals was recorded and at the end of 96 h was obtained the lethal concentration that affects 50% of the population (LC50-96h).

2.3 Mixtures action evaluation

The procedure for joint action evaluation of the mixtures was based on the method proposed for Marking (Marking, 1977). The method consists in obtaining the additive toxicity index of the mixture from the sum of individual toxicity of compounds using the following equations:

S= (Am/Ai) + (Bm/Bi)

Where: S = sum of individual toxicity of compounds; A and B corresponding to chemicals, in this case, DFB and PCA; i and m = LC50-96h of the single chemicals and mixture, respectively.

Additive Index **(AI) =** $\left(\frac{\mathbf{1}}{\mathbf{S}}\right) - \mathbf{1}$ when S≤ 1

AI= $\mathbf{S}(-\mathbf{1}) + \mathbf{1}$ when S ≥ 1

If the value of IA is 0, a simple additive effect is diagnosed; for IA< 0 the effect is antagonistic and for IA > 0 the effect is considered synergic.

After obtaining the AI, the Magnification Factor (MF) was calculated, demonstrating how many times the mixture is more or less toxic than the isolated compounds. The MF is calculated by adding 1 to the AI in the case of synergism and in the case of antagonism, the same procedure is performed with the absolute value of the AI and then the inverse of the value is calculated.

The isobologram was used to graphically display toxicological interaction (synergism, additivity or antagonism) for all tested binary mixtures of DFB and PCA. This was constructed by plotting concentrations of the pollutants that either, alone or in combination induced 50% lethality to fish (LC50) (Jonsson and Aoyama, 2007; Marking, 1977; Rand and Petrocelli, 1985).

2.4 Test for enzymatic evaluation

For enzymatic analysis, ten adult fish were exposed to sublethal doses of the compounds and mixtures thereof in the presence and absence of soil for 96h. The sublethal

doses were calculated from the LC50 values obtained in acute toxicity test (LC_{50}/10 - CL_{50}/50 and LC_{50}/100).

The procedures for this test were the same as described in acute toxicity tests. After 96 hours the animals were anesthetized with benzocaine (0.1 g/L) and sacrificed by spinal cord section for removal of the gills.

The organs were divided into equal portions, weighed and homogenized in the appropriate buffer for each enzyme at a ratio of 1:4 (weight/volume).

Then, this homogenate was centrifuged at 10.000xg, for 20 min, at 4°C and the supernatant was collected and maintained at -80°C before using for to determine enzymes activities and concentration protein

2.5 Activities enzimatic

The supernatant of gills tissue was used to quantitate the enzymes CAT, AcP, and, AlP.

The specific activity of CAT was determined according to the method described by Aebi (Aebi, 1984). 50 µL of the supernatant was added to 250 µL of reactant solution (0.03 M hydrogen H2O2 in 50 mM phosphate buffer at pH 7), after which the absorbance at 240 nm was monitored for 1 min. The specific activity of catalase was expressed in nmoles of H_2O_2.min^{-1}.mg $protein^{-1}$.

For determination of the AcP activity, 10 µL of supernatant was added to 15 µl of 100 mM acetate buffer (pH 5) and 125 µl of 10 mM p-nitrophenyl phosphate (pNPP) solution. AlP activity was measured as AcP, except for addition 15 µL of 250 mM glycine buffer (pH 9.4) and 10 µL of 20 mM $MgCl_2$ as a cofactor. The mixtures were incubated for 40 min at 37 °C, after which the reaction was halted by adding 50 µL of 1 M NaOH, followed by measuring the absorbance at 405 nm (Jonsson and Aoyama, 2007). The specific activity of AcP and AlP was expressed in nmoles pNP.min^{-1}.mg $protein^{-1}$. The analyses were performed in triplicate and employed a microplate spectrophotometer EON BioTek.

2.5.1 Concentration Protein

Protein concentration in the samples was determined in triplicate by the Lowry method (Lowry et al. 1951) at 560 nm, using bovine serum albumin as standard.

2.6 Statistical analysis

LC50-96h values were calculated using probit analysis (Statgraphics Plus v. 5.1 software) and were considered to be statistically different when there was no overlap of the 95% confidence intervals. GraphPad Prism v. 5.1 software was used for the other statistical analyses and data were presented as mean ± SD and analyzed by one-way analysis of variance (ANOVA). Dunnett test was used to verify differences between tested concentrations and control. Differences were considered significant at $P < 0.05$ or $P < 0.01$.

3. RESULTS

3.1 Acute toxicity

Results of the acute toxicity bioassays are summarized in Table 1. LC50-96h values greater than 100 mg/L were obtained for the DFB and the mixture containing 75% of this compound, in the presence and absence of soil. PCA presented higher toxicity to the fish both in the isolated form and in the mixtures with 50% and 75% of this compound. However, with soil presence, the median lethal concentration obtained for the PCA alone and in the mixtures with 50 and 75% PCA increased.

Table 1. Median lethal concentration (LC50-96h) of DFB, PCA, and mixtures thereof to tilapia fish in mg/L.

Conditions	DFB	PCA	75% PCA	50% PCA	25% PCA
Without Soil	> 100	25 (16.7-29.56)	15 (10.7-21.3)	40 (29.15-42.22)	> 100
With soil	> 100	37 (31.7-42.9)	25 (23.8-27.2)	45 (43.9-49.8)	> 100

The confidence intervals of 95% are in parenthesis

.

3.2 Evaluation of mixtures action

The range of the Additive Index was calculated by a system provided by Marking & Dawson (Marking, 1977) in order to judge whether the Additive Index values are different from zero. So, mixtures that resulted in ranges for the index that overlapped zero were judged to be only in the additive in toxicity. In the other hand, ranges that did not overlap zero were either greater (synergism) or less than additive (antagonism) (Rand and Petrocelli, 1985).

According to Marking's Additive Index (AI), the mixtures that showed antagonistic toxicity without soil were 50% of PCA (AI = -0.033) and 25% of PCA (AI = -0.77). What means that the toxic effect of these mixtures was 0.96 and 0.57 times lower, respectively, that expected when looking at the toxicities compounds individual. However, only the range of the mixture with 25% of PCA did not overlap zero and for this reason was considered an actual antagonist effect (Table 2).

In the presence of soil, the mixture with 25% PCA continued to show significant antagonistic interaction with DFB (AI = -0.42), being 0.7 times less toxic. Nevertheless, also in soil presence, the mixture 75% PCA showed a light synergic interaction (AI = +0.69) between the compounds that was significant. A more than additive interaction according to the index value (AI = +1,02) was also observed for the same mixture in soil absence (Table 2).

Table 2. Additive Index (AI) and Magnification Factors (MF) calculated after exposure of tilapia (n=10) to different proportions of the mixtures of DFB and PCA.

	75%PCA		**50%PCA**		**25%PCA**	
Conditions	AI	MF	AI	MF	AI	MF
Without soil	+1,02 (2.33 to 0.03) [1]	2.0**	-0,033 (-0.47 to 0.57)	0.96	-0,77 (-0.59 to -1.24)	0.57*
With soil	+0,69 (1.01 to 0.41)	1.7**	+0,19 (0.37 to -0.03)	1.2	-0,42 (-0.33 to -0.53)	0.70*

[1] Range of AI in parentheses, MF = times and * significant antagonism; ** significant synergism

The meaning of AI values is demonstrated as the points in the isobolograms Fig. 1 and Fig. 2. Therefore, a point over the solid line denotes additive effect while a point above or bellow this line means antagonism and synergism, respectively.

The isobologram was used as a graphical representation of synergism and antagonism of compounds without mathematical derivation. This graphical representation showed LC50-96h and which compound concentration was needed to achieve this the effect

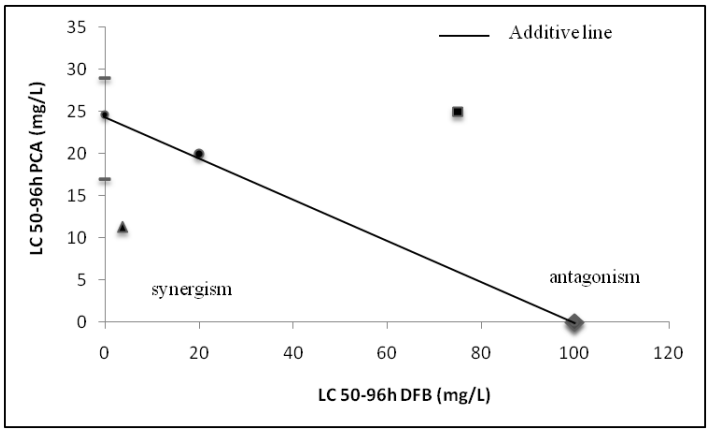

Fig. 1. Isobolographic analysis of mixtures of DFB and PCA without soil. The additive line is the zero-interaction isobole constructed from LC50-96h with each compound alone. LC50 values for 75% PCA (▲), 50% PCA (●), and 25% PCA (■).

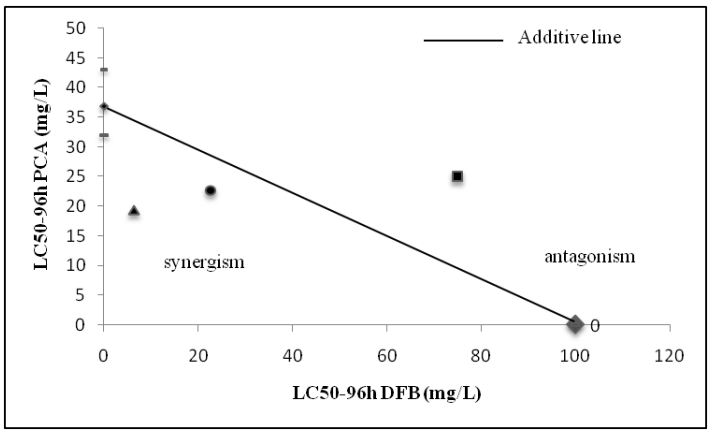

Fig. 2. Isobolographic analysis of mixtures of DFB and PCA with soil. The additive line is the zero-interaction isobole constructed from LC50-96h with each compound alone. LC50 values for 75% PCA (▲), 50%PCA (●), and 25%PCA (■)

3.3 Activities enzimatic

The enzyme catalase (CAT) showed several increases in its activity in the absence of soil for the isolated compounds and their mixtures. However, 2 mg/L of DFB alone (Fig. 3) and in the mixture with 25% of PCA (Fig. 3) caused a decrease of 35% and 21%, respectively, in CAT activity. A decrease of 43% was also observed for 0.8 mg/L of the mixture with 50% of each compound (Fig. 3).

In the presence of soil, CAT showed only decreases in its activity, being the highest of 80% observed in 0.45 mg/L of the mixture with 50% of PCA (Fig 3).

Acid phosphatase (AcP) in the absence of soil showed a decrease in activity for the isolated compounds (Fig. 4) and in the mixture with 25% PCA (Fig 4), but in the mixture, with

75% PCA, significant increases were observed in the activity of this enzyme (Fig 4). However, in the presence of soil, increases in AcP activity were observed for 1 and 10 mg/L of PCA alone, in the other mixtures, the enzyme had a decrease in activity.

Alkaline phosphatase (AlP) in soil absence showed significant decreases of 36%, 58% and 62% for 0.15, 0.3 and 1.5 mg/L of the mixture with 75% PCA (Fig 4), respectively, and increases of 177%, 147% and 45 % to 0.4, 0.8 and 4 mg/L of the mixture with 50% PCA, respectively (Fig 4). In the presence of soil, the highest increase in AlP activity was 55% observed in 3.7 mg/L of PCA alone (Fig 4) and the highest decrease was from 47% to 0.5 mg/L of 75% PCA (Fig 4).

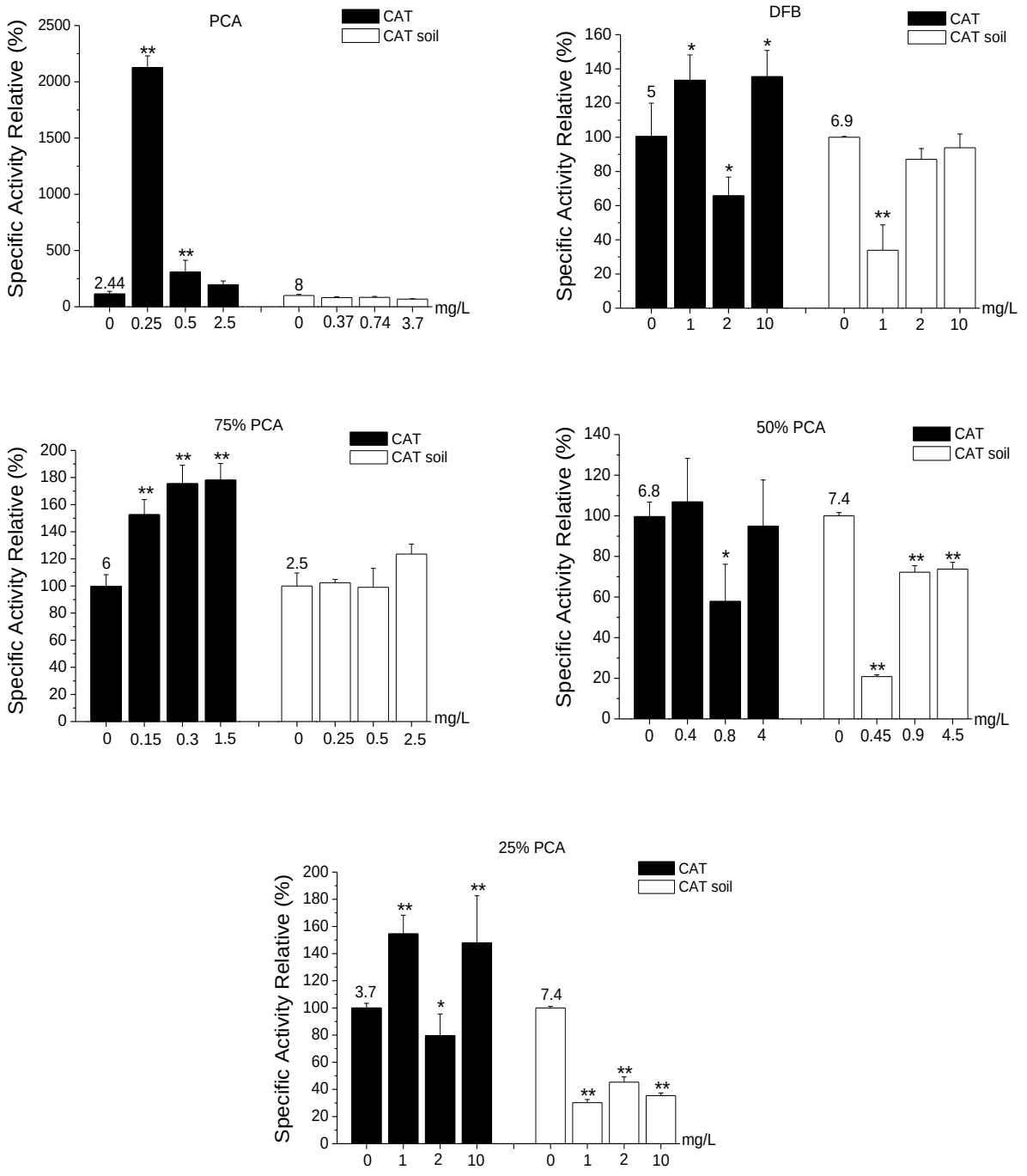

Fig. 3. Effect PCA, DFB, and mixtures thereof on Catalase (CAT) activities of tilapia gills (n = 10) after 96h of exposure, in presence and absence soil. The numbers that appear on the control bars (zero concentration) referring to 100% activity correspond to nmol of H_2O_2 decomposed per min per mg of protein. *Significantly different from control at P<0.05 and ** P<0.01. Data are expressed as mean (±standard deviation).

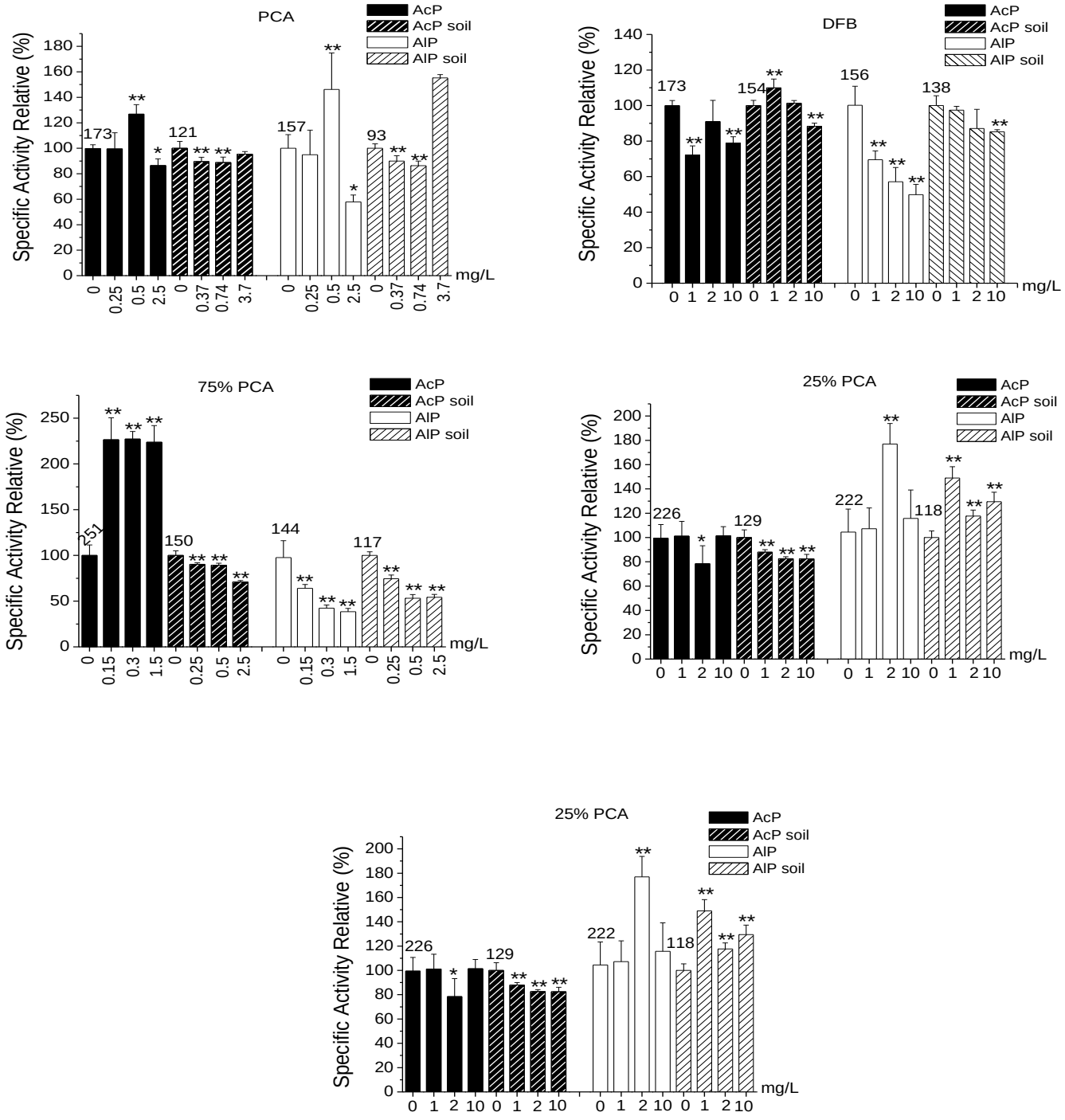

Fig. 4. Effect PCA, DFB, and mixtures thereof on Acid Phosphatase (AcP) and Alkaline Phosphatase (AlP) activities of tilapia gills (n = 10) after 96h of exposure, in presence and absence soil. The numbers that appear on the control bars (zero concentration) referring to 100% activity correspond to nmol of p-nitrophenol produced per min per mg of protein. *Significantly different from control at P<0.05 and ** P<0.01. Data are expressed as mean (±standard deviation).

4. DISCUSSION

As can be seen in Table 1 the presence of soil in the acute toxicity test decreased the bioavailability of the compounds DFB and PCA in water. Xenobiotics in aquatic environments may be partitioned between the aqueous phase and the soil particles (Hartman and Martin, 2000). This partitioning depends on the physical and chemical properties of the water, the type of soil present, and the molecular nature of the xenobiotic (Hartman and Martin, 2000; Jonsson and Nunes Maia, 1999). Thus, this partitioning can affect the availability of the xenobiotics to different aquatic organisms.

For diflubenzuron and p-chloroaniline the water solubility is low with 0.089 and 0.039 mg/L at 20°C, respectively, and with high Log Kow constants of 3.83 and 1.88 respectively, is expected that these compounds are associated with soil particles (Kishida and Otori, 1980; Schaefer et al., 1979). Besides these factors, DFB and PCA may bind to soil particles with high amounts of organic material under aerobic conditions and low pH levels (Gawlik et al., 1998; Ole B Samuelsen, 2016). Some authors have studied the bioavailability of DFB and PCA in soil and organic matter, but there is no data on the mixture of these compounds (Samuelsen et al., 2015; Steinberg et al., 1993).

The presence of soil did not change the type of interaction that occurred between the compounds in the mixtures (Fig 1 and 2). The exposure of organisms to the mixture with 25% of the PCA resulted in a less severe response to fish than the compounds applied singly, (antagonistic effect). The antagonistic interaction that occurred between the DFB and PCA would not minimize the toxic effects that this mixture may cause in the aquatic environment. For example, the DFB of this mixture in the aquatic environment can be degraded or metabolized and form more p-chloraniline and as can be seen in Table 2, the mixture containing the major part of p-chloroaniline (75%) showed a synergistic effect. Therefore, knowledge of the toxic effects of mixtures is very important for the establishment of maximum permissible levels of the xenobiotics in the aquatic environment. Several authors have studied interactions between various xenobiotics, including metals, pesticides and other organics pollutants that represent serious risks to many aquatic organisms (Ensenbach and Nagel, 1995; Jonsson and Aoyama, 2007; Jonsson and Nunes Maia, 1999; Mofeed and Mosleh, 2013; Pérez et al., 2013; Zhang et al., 2010). However, there is a gap in the scientific literature on the environmental impact of the mixture of the DFB and its PCA metabolite in aquatic organisms.

The observed interactions between DFB and PCA in the mixtures suggest that Marking's Additive Index (AI) was adequate to predict the combined toxicity of the compounds.

Other studies have also used the Marking's Additive Index to provides a prognostic of the toxic effect of the mixtures of the different compounds to organisms and environmental (Henry et al., 1994; Jonsson and Aoyama, 2007). Using the Additive Index concept, the mixtures of two different herbicides presented synergistic action in presence of a surfactant and antagonism in absence of it (Green and Abdelghani, 2004). The combination of insecticides malathion, methyl parathion, endosulfan with herbicide 2, 4-D showed strictly additive toxicity to rohu fish (Nair et al., 2007).

This research showed that sublethal doses of DFB, PCA, and their mixtures were able to alter important enzymes present in fish metabolism. Enzyme analysis of organs such as the gills can provide important information about the fish health (Boeger et al., 2003). In fish, gills are the first organs to come into direct contact with aquatic xenobiotics and possess a detoxification system (Kubrak et al., 2012).

Numerous pesticides can induce an increase of reactive oxygen concentrations in cells, and consequently the development of antioxidative defenses such as catalase (Coelho et al., 2011; Hamed et al., 2016; Husak et al., 2014). The CAT activity after exposure of tilapia for 96 h to compounds isolated and their mixtures had a significant increase in absence soil. This increase indicated excessive production of H_2O_2 in cells of gills and that the CAT stimulation was initiated to combat oxidative stress (Mofeed and Mosleh, 2013). When occurs an excessive increase in Oxygen-Reactive Species (ROS) and oxidative stress in response to acute intoxication with compounds, the activity of CAT can decrease. It was reported that catalase is sensitive to free radicals, in particular, superoxide anion and hydrogen peroxide, which can inactivate the enzyme (Kubrak et al., 2012). This may explain the decrease in CAT activity in the presence of soil and the excessive increase in ROS may have been caused by the ingestion of the soil particles with the compounds adsorbed by the fish. Hartman and Martin, (1984), observed that the compound glyphosate ingested along with particulate matter by the microcrustacean *D. pulex* was more toxic by this route of exposure.

The enzyme CAT has been widely used as a biomarker of environmental pollution for being sensitive to various xenobiotics and easy to be measured. *P. lineatus* fish exposed to higher concentrations herbicide clomazone presented activation of the antioxidant enzyme CAT (Pereira Maduenho and Martinez, 2008b). Tilapia exposure to insecticide methomyl for 30 days showed significant increases in CAT, suggesting the presence of oxidative stress (Meng et al., 2013). A decrease of catalase (CAT) activity in gills of the tilapia exposed to samples of water from Monjolinho river contaminated with metals was observed (Carvalho et al., 2012).

Phosphatases are hydrolytic enzymes that catalyze the dephosphorylation of a wide variety of orthophosphate esters and transphosphorylation reactions (Aoyama et al., 2003). These enzymes are associated with carbohydrate metabolism, oxidative phosphorylation as well as cell growth and differentiation (Moog, 1946). AlP is a membrane-bound polyfunctional enzyme (Molina et al., 2005), that plays important functions like in protein synthesis, secretory activity, spermatogenesis, glycogen metabolism and membrane transport activities (Moog, 1946; Thirumavalavan, 2010). Acid phosphatase is a lysosomal hydrolase enzyme that in situations of cellular stress can occur its extravasation to intra and extracellular fluids besides participating in the process of cellular autolysis (Kumaresan and Karuppasamy, 2011; Suresh et al., 1993). Thus, any change in activity these enzymes can affect the health of an organism in some way (Fırat et al., 2011; Palanivelu et al., 2005).

The compounds DFB, PCA, and mixtures thereof increased and decreased the activity phosphatases of the gills in the presence and absence of soil. Tilapia like other teleost fishes breathe through the gills and (Kumaresan and Karuppasamy, 2011)increased phosphatase activity in this organ may indicate rupture of the cell membranes and lysosomes (Kumaresan and Karuppasamy, 2011; Lohner et al., 2001; Thirumavalavan, 2010). The gills are in direct contact with the DFB and the PCA in the water and these compounds can inactivate the phosphatases reducing their activity by this route exposure (Mir et al., 2016; Patrícia Carraschi et al., 2012). On the other hand, the reduction in the phosphatase activity can be due histopathological changes in the gills exposed to the compounds (Mir et al., 2016; Rs and Shaikh, 2013).

There are a number of studies on the changes in the AcP and AlP enzymes of the organs of fish exposed to differents toxicants. The acid and alkaline phosphatase activity increased in the gill of *Oreochromis mossambicus* when exposed to cadmium chloride (Thirumavalavan, 2010). A significant decrease in acid phosphatase activity in the liver of the *Channa Punctatus* fish was observed after exposition to insecticide Malathion (Rs and Shaikh, 2013). Similar observations were noted in alkaline phosphatase activity in muscle and gill of *Heteropneustis fossilis* fish on exposure to insecticide dimethoate, except acid phosphatase of gills which showed increased activity (Borah and Yadav, 1996).

5. CONCLUSION

Our results showed that DFB, PCA, and mixtures thereof can affect enzymes important for fish health. The mixture containing 75% of the toxic compound PCA showed synergistic action for the organisms. In this case, these products should be used and discarded

with caution, since once in the aquatic environment they may affect other organisms and human health through fish meat and contaminated water.

The presence of soil showed that the compounds could be adsorbed on their particles, but the toxic interaction between them remained the same. Our results provide important data that can be used to predict aquatic environments contaminated with these pollutants.

CAPÍTULO III

ESTUDOS COM ZEBRAFISH *(Danio rerio)*

Artigo submetido para publicação: Dantzger, D.D. et al.

Diflubenzuron, p-chloroaniline, and, mixtures thereof impact on zebrafish early life stages and adults.

Aquatic Toxicology.

ABSTRACT

The insecticide diflubenzuron (DFB) is intensively used by fish farmers to control parasites that cause disease in fish and, when degraded or metabolized, generates the highly toxic metabolite p-chloroaniline (PCA). These compounds are present in mixtures in the aquatic environment and fish are vulnerable and heavily exposed to these mixtures. This study investigated the toxic effects of DFB, PCA, and mixtures with 75%, 50% and 25% PCA on zebrafish early life stages and adults, and the parameters as mortality, hatching, abnormalities in embryonic development, morphological and histological changes were evaluated and reflected acute toxicity and sublethal effects. The acute toxicity tests showed that mixtures of DFB with PCA were more toxic to fish than insecticide itself. Among the mixtures evaluated 75% of PCA caused mortality of organisms, followed by 50% and 25% PCA. The embryos with 1hour post fertilization (hpf) exposed to mixtures of DFB and PCA for 96 h presented a delay in hatching and abnormalities such as pericardial edema, yolk sac edema, and, spine malformation. The larvae (144 hpf) exposed to PCA (1.35mg/L) and of the mixtures with 50% PCA (1.56mg/L) and 75% PCA (1.26mg/L) have changes in liver morphology, gut, and swim bladder. The presence of DFB decreased the toxic effects of PCA to the organisms and the mixture containing 25% of PCA showed antagonistic effects. The range of endpoints used on the embryo and larval test contributes to a better knowledge of the toxicity of the compounds DFB, PCA, and their mixtures.

1. INTRODUCTION

Many agrochemicals have been used in fish farming to control parasites that cause disease in fish such as the crustaceans *Lernaea cyprinacea* and *Dolops carvalhoi*, among others, due to the lack of products for this purpose (Coelho et al., 2011; Gabriel et al., 2012; Guimarães et al., 2007; Pereira Maduenho and Martinez, 2008). The agrochemical diflubenzuron (DFB) is an insecticide benzoylphenylurea derivative, (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea), intensively used by fish farmers because it inhibits the chitin synthesis of parasites causing their death and it is not lethal for fish (Branson et al., 2000; Haya et al., 2005; Henrique et al., 2005; Ole B. Samuelsen, 2016). However, this insecticide is extremely toxic to other aquatic organisms that are part of the food chain, like the microcrustacean *Daphnia similis* (Jonsson et al., 2015; Macken et al., 2015). This compound in the environment can suffer photodegradation, hydrolysis or metabolization, producing several metabolites, among them, the p-chloroaniline (PCA) (He et al., 2013a; Olsvik et al., 2013). PCA is widely used industrial chemicals and highly toxic to a variety of aquatic organisms, including fish, and humans, so it is considered a priority pollutant in the environmental risk assessment (Kataoka, 1996; Sihtmäe et al., 2010). DFB and PCA can indirectly contaminate the water bodies through spraying in agriculture, surface runoff or industrial effluents (Ensenbach and Nagel, 1995; Pérez et al., 2013; Sihtmäe et al., 2010).

These compounds, like a wide variety of xenobiotics, are present as mixtures in the aquatic environment and data used in ecotoxicological risk assessment are predominantly based on single substance evaluation (Authman et al., 2015; Schreiner et al., 2016). The effects of the compounds mixtures are known to be different from those predicted on the basis of the individual compounds effects, so, single substance evaluation may underestimate data with ecological relevance (Backhaus et al., 2013; Gregorio et al., 2013).

While effects of DFB exposure on various organisms have been well documented, there is no data with respect to the impact of their metabolite PCA combinations in aquatic environmental (Coelho et al., 2011; Gabriel et al., 2012; Guimarães et al., 2007; Pereira Maduenho and Martinez, 2008). The impacts of the mixture of DFB and PCA are of ecological concern, particularly during an organism's sensitive, developmental life stages. The toxicological effects of this mixture on the health of the early stages of fish life are largely unknown, so this research becomes essential since fish are widely used as sentinels for assessing aquatic pollutants (Hill et al., 2005; Parng et al., 2002). In this regard, this research was used zebrafish as an in vivo model organism because of its unique features, including high fecundity,

embryo transparency, fast and well-characterized development, low cost, similarity to the human genome, short reproduction time, etc (Nagel, 2002; Parng et al., 2002). Compared with other vertebrates, the embryonic development of zebrafish is rapid, after 5 days fertilization, all the internal organs, including heart, liver, kidney, and pancreas are fully developed and functioning (Zhang et al., 2003). Many toxicological endpoints like anomalies, egg coagulation, tail extension and heart function can be evaluated a few days after fertilization. Finally, the zebrafish larva has the body transparent and changes in color and morphology of tissues like the liver and can be visually assessed without the need for surgery (McGrath and Li, 2008; Parng et al., 2002).

The present study analyzed the toxic effects of different proportions of the mixtures of DFB and PCA in zebrafish embryos, larvae and adults. The parameters as mortality, hatching, abnormalities in embryonic development, morphological and histological changes in some larvae organs were evaluated and reflected acute toxicity and sublethal effects.

2. MATERIALS AND METHODS

2.1 Chemicals

Diflubenzuron (trade name dimilin®) contained 25% of the active ingredient and 75% of inert excipients was purchased from Chemtura Industria Quimica Ltda (Brazil), and, p-chloroaniline (PCA) was supplied by Sigma-Aldrich, Brazil, technical grade, purity > 99%. Chemicals were tested individually and in mixtures with the proportions: 75%, 50% and 25% PCA. The test solutions were prepared with carbon-filtered water.

Analysis of compounds concentrations and the renewal of the test solutions were not performed in the assays because the degradation time of PCA and DFB in water is 6 and <7 days at temperature 28°C and pH 7.7, respectively (Ekici et al., 2001; Zaidi et al., 2013)

2.2 Bioassays

2.2.1 Maintenance of adult zebrafish

Zebrafish *(Danio rerio*) were obtained from a local supplier (Agrosete, Sumaré, São Paulo, Brazil) and culture established in the laboratory, at the Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas (UNICAMP). Adult fish are maintained in glass aquaria containing 10L carbon-filtered water at 28 ±2ºC.The physical and chemical parameters of the water were monitored and remained constant: pH 7.4 ± 0.4; dissolved oxygen 8 ± 2 mg O_2/L; conductivity 160 ± 5 µS/cm and hardness 50 ± 2 mg $CaCO_3$/L.

Fish were exposed to natural light-dark cycle and fed twice a day with commercial feed TetraMin Plus (Tetra Holding US Inc.) supplemented once a day with brine shrimp (Artemia spp.). These fish were used to obtain embryos and in acute toxicity tests.

2.2.2 Reproduction and Egg acquisition

For reproduction, zebrafish was transferred to spawning aquarium with the bottom covered with glass marbles to protect the eggs from being eaten and a partition with holes that allowed the passage of water from one side to the other of the aquarium and the males remained separated from the females (ratio 1:2) until mating. The genitors stayed in this mating aquarium overnight in the dark, and in the following morning, the light was turned on, and the isolation partition was removed triggering the spawning. Half an hour after mating, eggs were collected and observed using a stereomicroscope (Bel photonics®) and the fertilized eggs (embryos with normal development of a blastula) were used for exposure to DFB, PCA and their mixtures.

All procedures used in the experiments with fish were approved by the Ethics Committee for the Use of Animals of the State University of Campinas (CEUA/UNICAMP) under the registration Nº 3641-1 (Law Nº 11794/2008).

2.2.3 Embryonic development assays

Forty-eight embryos of zebrafish with 1hour post-fertilization (1hpf) were used per concentration tested and individually distributed in 24-wells microplates with 2 ml of test solution in each well (24 embryos per microplate, and two replicas per concentration). From each 24-wells microplates, four wells were internal controls (one embryo per well with 2mL filtered water) (Oecd/Oecd 2013). Embryos were exposed for 96 h to 2.2, 4.4, 9.7, 21.3, 46.8 and 100 mg/L of diflubenzuron, p-chloroaniline, and their mixtures that following proportions of 75%, 50% and 25% PCA. Test solutions were prepared with dechlorinated water (pH 7.0±0.5 and conductivity 160 ± 5µScm^{-1}) and the temperature during the test was 28±2ºC (Oecd/Oecd 1992).

Theses concentrations were chosen according to DFB and PCA concentrations tested on several fish species (Braunbeck et al., 1990; Bresch et al., 1990; Fraysse et al., 2006; Pereira Maduenho and Martinez, 2008b)

The embryos were observed in the inverted microscope (Nikon Eclipse TS100) with X40 magnification in 24 and 48 hours, whereas the larvae were observed in the stereomicroscope Bel photonics® with the magnitude of 2X in 72 and 96 hours. Endpoints as embryo and larvae abnormalities (pericardial edema, yolk sac edema, and spine malformation),

mortality and hatching were recorded. The assay was based on the OECD guideline on Fish Embryo Toxicity Test (FET) (Oecd/Oecd 2013).

The percentage of mortality, hatching, and, abnormalities were calculated after the data collection.

2.2.4 Assessment of acute toxicity for larvae and adult zebrafish

For the acute toxicity test with the larvae, 48 organisms with 96 hours post fertilization (hpf) were exposed to the compounds and their mixtures in two 24-wells microplates under the same design and conditions described in the embryonic assay (Oecd/Oecd 2013). For the acute toxicity test with adult zebrafish, (2±1cm, 6 months old), 10 organisms were transferred to glass aquaria containing 10 L of the test solution (5 fish per aquarium and two replicas per concentration) and this system remained static under constant aeration and controlled temperature (28 ± 2°C) (OECD, 1992). The test concentrations evaluated of diflubenzuron, p-chloroaniline, and their mixtures were 2.2, 4.4, 9.7, 21.3, 46.8 and 100 mg/L (OECD, 1992). The total period of exposure to test concentration was 96 hours, during which fish and larvae were not fed. At the end of the exposure period, the number of fish and larvae dead was registered to determine LC50-96h.

2.2.5 Morphological and histological assays

In the morphological tests, 10 Larvae with 144 hpf were exposed to sublethal concentrations of the DFB, PCA and their mixtures by 96h. Sublethal concentrations were equivalent to LC50-96 h /10 and are shown in Table 1.

After 96h, larvae were fixed alive with xanthine gum 1% on slides and morphological changes in the liver, gut and swim bladder in relation to larvae control were evaluated under inverted microscope Nikon Eclipse TS100 with 40X magnification and stereomicroscope BEL photonics® with 2X magnification (McGrath and Li, 2008; Zhang et al., 2003).

Table 1. Sublethal concentrations of PCA, DFB and mixtures thereof used in morphological and histological tests with larval zebrafish.

Compounds/Mixtures	LC50-96h/10 (mg/L)
PCA	1.35
DFB	8.12
75% PCA	1.26
50% PCA	1.58
25% PCA	3.3

In the histological tests, 10 larvae with 144 hpf were exposed to sublethal concentrations (Table 1) of the DFB, PCA and their mixtures by 96h. Following, the larvae were anesthetized by a tricaine methanesulfonate solution (MS-222, Sigma) at 0.3 g/l. and fixed by immersion in 4% v/v paraformaldehyde in PBS, pH 7.4 for 24 hours at 4ºC. After fixation, paraformaldehyde was removed with five washes of 5 minutes in PBS and larvae were embedded in a mixture of agar 1.5% and sucrose (Panreac) 10% in PBS. After the mixture was solidified, the larvae were cryoprotected in a 30% w/v sucrose solution in PBS for 24 h. Agar blocks containing cryoprotected larvae were frozen in a cryostat (Leica CM 1850) and then cut at -28ºC in 10-µm-thick transversal serial sections, which were collected on slides. Histological sections were stained with hematoxylin-eosin to observe possible morphological changes. For each larva, about thirty slide sections were obtained. Ten larvae were used for each group. The slides were photographed at ×100 magnification using a microscope (Leica) coupled to a digital camera (OptikamB3) (Prieto et al., 2014).

2.3 Statistical analysis

LC50-96h values for early-life stages and adults were calculated using probit analysis (Statgraphics Plus v. 5.1 software) and were considered to be statistically different when there was no overlap of the 95% confidence intervals. GraphPad Prism v. 5. software was used for the other statistical analyses and data were presented as mean ± SD and analyzed by one-way analysis of variance (ANOVA) and the Dunnett test was used to verify differences

between tested concentrations and control. Differences were considered significant at $P < 0.05$ or $P < 0.01$.

3. RESULTS

3.1 Embryonic development assays

To evaluate the possible toxicity of different concentrations of the DFB, PCA, and mixtures thereof on embryonic development of zebrafish, we analyzed the hatching rate, mortality, and abnormalities during at 96 h of exposure**.** Control embryos and those exposed to isolated DFB and PCA started to hatch with 72 hpf and a delay in hatched (96hpf) was observed in the embryos exposed to concentrations of the mixtures with 75%, 50% and 25% of PCA (Fig.1).

In the first 24 hpf, mortality of the embryos exposed to the isolated compounds was observed. About 10% and 20% of embryos were dead at concentrations of 46.8 and 100 mg/L of DFB in the first 24 hpf. The number of dead embryos exposed to PCA in the first 24 hours was higher than for DFB and increased as the concentration increased, except for the concentration of 21.3 mg/L (Fig.1). The mixtures with 75% and 50% of PCA caused embryo mortality after 48 h of exposure to the mixtures and no embryo dead was observed in the mixture with 25% PCA during 96 hpf and 100% of embryos was hatched for this mixture (Fig.1).

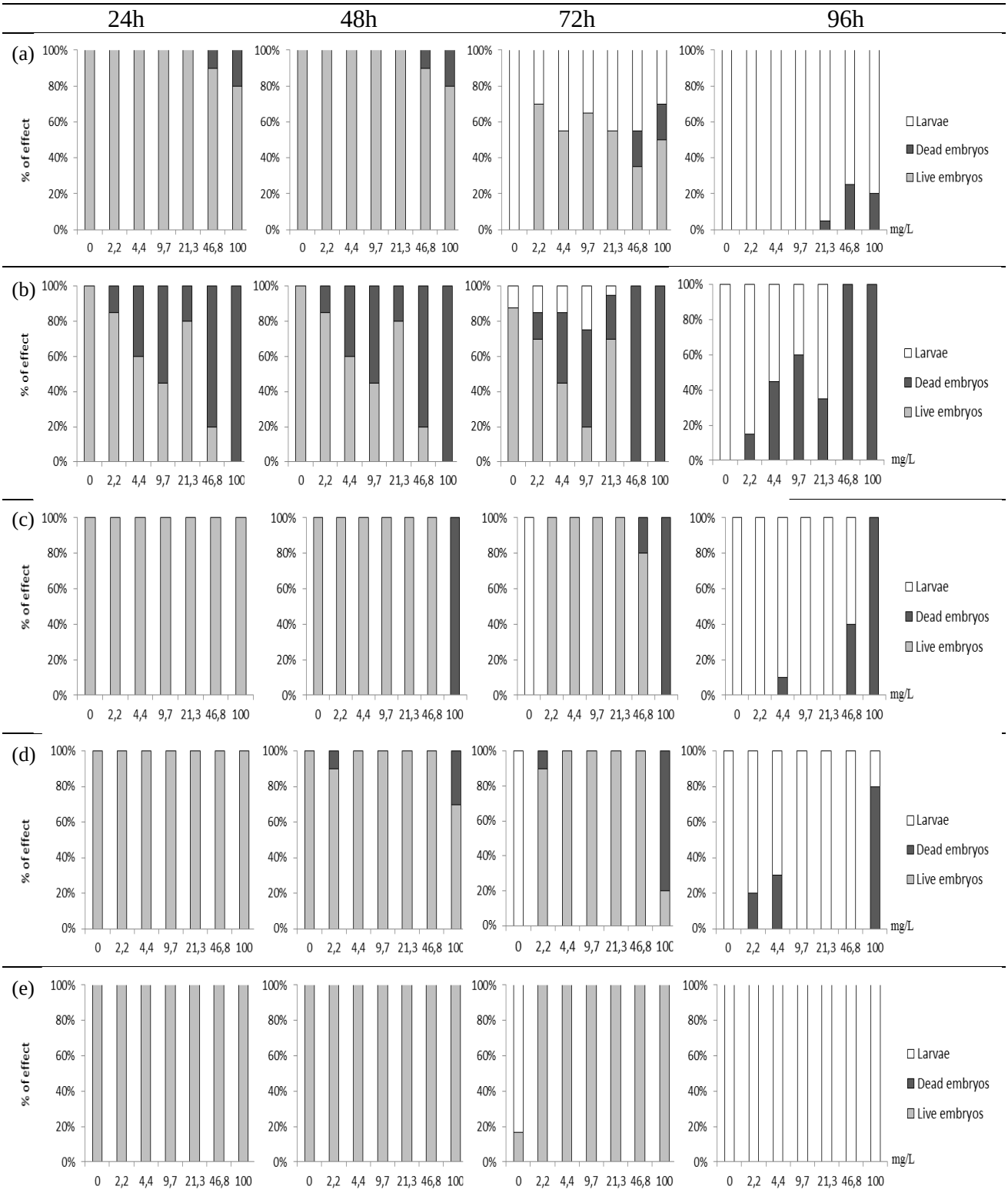

Fig. 1. General overview of DFB (a), PCA (b), 75% PCA (c), 50% PCA (d) and 25% PCA (e) effects on zebrafish embryo and larvae (n=48) during the 96 h of exposure. Black bars mean proportion of embryos that died, gray bars, embryos stayed alive and white bars that hatched. All concentrations are significantly different from the control ($p < 0.05$).

Some embryos exposed to the compounds and their mixtures by 96h had various malformations that did not allow their survival or hatching. Others succeeded to hatch and become larvae, but they did not survive due to various abnormalities that occurred during their embryonic development. The percentages of embryos with malformations and of larvae with abnormalities for each concentration of compounds and their mixtures can be observed in Table 2.

Embryos with malformations (division and abnormal cell growth) were observed in some concentrations of PCA isolated and in the mixtures with 75% and 50% thereof. However, the embryos exposed to the various concentrations of the mixture with 25% of PCA had normal development and the larvae showed no morphological changes.

Larvae with abnormalities were observed at concentrations of the isolated compounds and in the mixtures with 75% and 50% of PCA. Spine malformation, pericardial edema, and yolk sac edema were the main morphological changes observed in embryos exposed to isolated PCA and to mixtures with 75% and 50% of PCA. The embryos exposed to isolated DFB presented the same morphological changes with exception of the yolk sac edema.

Zebrafish embryos and larvae with normal and abnormal development after 96h of exposure to DFB, PCA, and their mixtures can be observed in Fig. 2.

Table 2. Abnormality rate (%) observed in zebrafish embryos and larvae exposed to different concentrations of DFB, PCA, and mixtures thereof for 96 h. Data are expressed as mean (±standard deviation) (n = 48).

	% Abnormalities			
	Embryos	Larvae		
Concentrations mg/L	**Malformations***	**Pericardial edema**	**Yolk sac edema**	**Spine malformation**
DFB				
0	0	0	0	0
2.2	0	0	0	0
4.4	0	0	0	10 (±1.5)
9.7	0	5 (±0.5)	0	0
21.3	0	0	0	0
46.8	0	0	0	0
100	0	0	0	0
PCA				
0	0	0	0	0
2.2	0	0	0	6 (±1.2)
4.4	0	0	9 (±1)	0
9.7	8.3 (±1.1)	0	0	0
21.3	42.8 (±2.3)	7.7 (±0.5)	7.7 (±0.5)	23 (±1)
46.8	5 (±0.5)	0	0	0
100	0	0	0	0
75%PCA				
0	0	0	0	0
2.2	0	0	0	0
4.4	100 (±5)	0	0	0
9.7	0	0	0	0
21.3	0	0	0	0
46.8	50 (±3)	16 (±2)	67 (±5)	17 (±1.5)
100	100 (±1)	0	0	0
50%PCA				
0	0	0	0	0
2.2	1 (±5)	0	0	0
4.4	0	0	0	0
9.7	0	0	0	0
21.3	0	0	0	10 (±0.5)
46.8	0	30 (±5)	20 (±3)	50 (±3)
100	100 (±15)	0	0	0
25%PCA				
0	0	0	0	0
2.2	0	0	0	0
4.4	0	0	0	0
9.7	0	0	0	0
21.3	0	0	0	0

46.8	0	0	0	0
100	0	0	0	0

*** Division and abnormal cell growth**

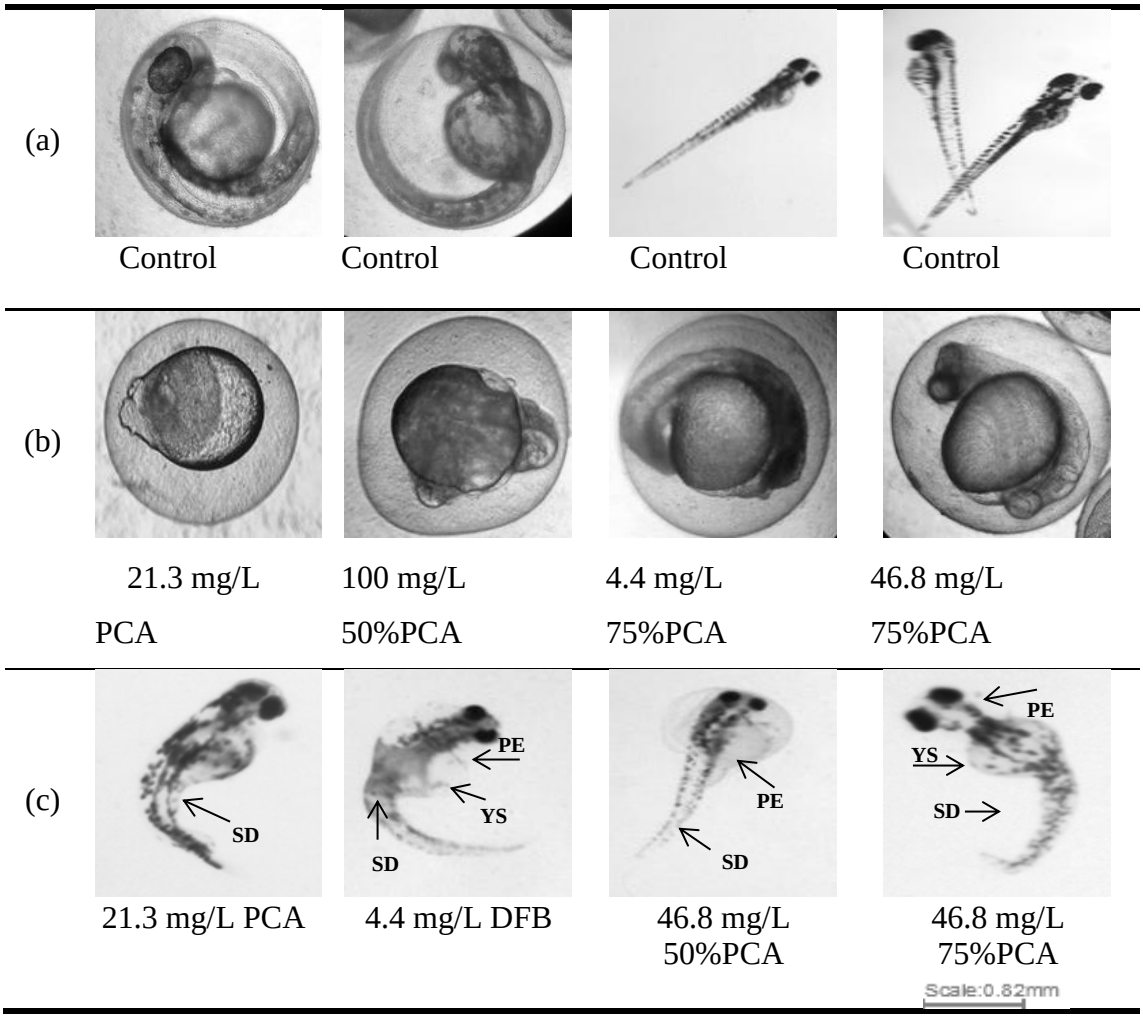

Fig. 2. (a) Zebrafish embryos and larvae control with normal development, (b) Embryos, (c) Larvae with abnormalities observed after 96h of exposure to DFB, PCA, and their mixtures. SD= Spine Deformity, YSE= Yolk-Sac Edema, PE= Pericardial Edema.

3.2 Assessment of acute toxicity for larvae and adult zebrafish

Among the organisms exposed to DFB, PCA, and their mixtures for 96h in the acute assay, the larvae were most susceptible than adult zebrafish.

DFB showed no toxicity to adult fish, but about 80 mg/L of this compound was able to kill 50% of fish larvae.

PCA was more toxic to larvae (LC50-96h = 13 mg /L) and adult fish (LC50-96h = 19) than DFB and mixtures studied. Among the proportions of the mixtures evaluated, 25% of PCA was less toxic to organisms, followed by mixtures with 50% and 75% of PCA.

Percentage of larval and adult mortality of zebrafish of the acute toxicity bioassays can be observed in Fig.3

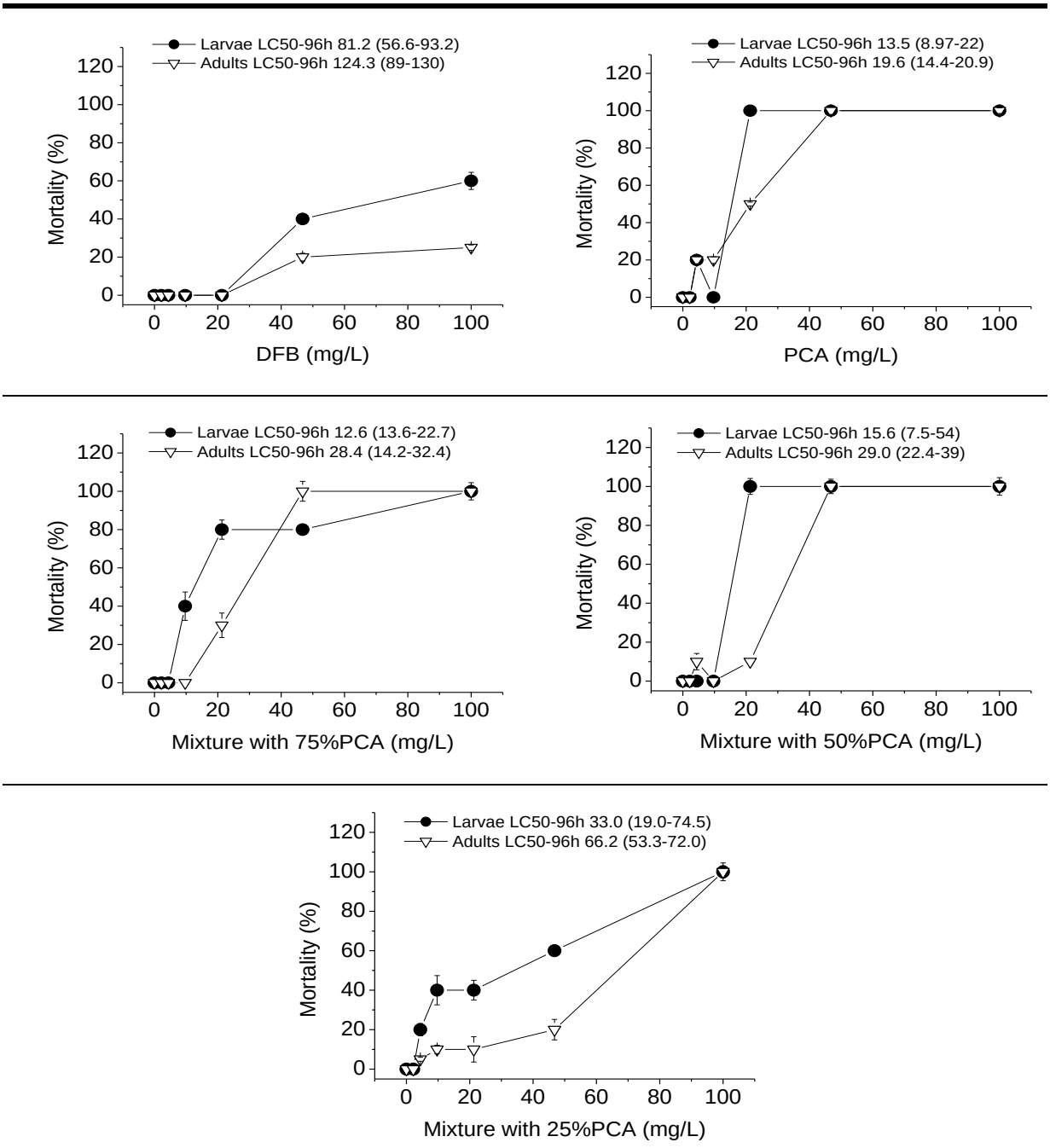

Fig. 3. Percentage of larval (n=48) and adult (n=10) mortality of zebrafish (mean value ±standard error) exposed to different concentrations of DFB, PCA and their mixtures for 96 hours.

3.3 Morphological and histological assays

Zebrafish larvae (144 hpf) exposed to DFB, PCA, and mixtures thereof showed signs of hepatotoxicity and changes in the swim bladder and gut that was possible to be visualized by using an inverted microscope.

As shown in Fig. 4a, untreated zebrafish larvae exhibited liver, swim bladder, and gut with regular contours and characteristic colors, whereas the liver and the gut of treated larvae displayed amorphous, greenish yellow coloration and/or uninflated swimming bladder (Fig. 4b).

To assess additional liver morphological defects, hepatic cellular morphology was examined by histology (Fig. 4c). In 1,35 mg/L of PCA and 1,56 mg/L of the mixture with 50% of PCA the hepatocytes had increasing cytoplasmic and decrease nucleus-to-cytoplasm ratio (Fig.4e/4f) compared with control (Fig. 4d). The larvae with abnormal hepatocytes was scored in at least 3 clutches with an average n= 10 per group and $P < 0.05$.

One hundred percent of the larvae exposed to 1.35 mg/L of PCA and 80% of the larvae exposed to 1.56 mg/L of the mixtures with 50% of PCA presented changes in the liver, gut and swim bladder (Fig. 5). In mixture with 75% of PCA, 80% of larvae showed a failure to inflated swim bladder (Fig. 5). Larvae exposed to sublethal concentration of DFB and the mixture with 25% of the PCA showed normal phenotypes.

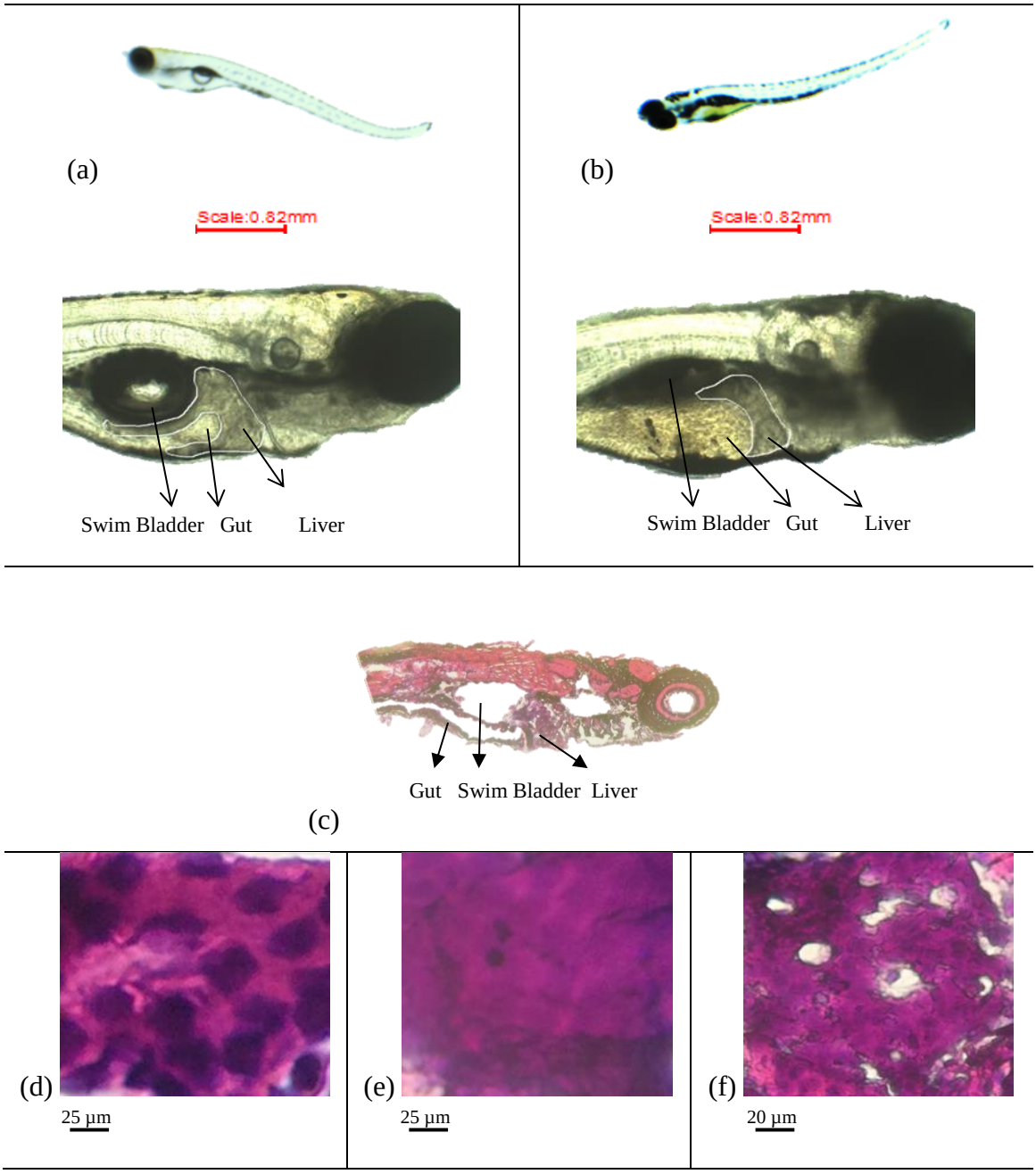

Fig. 4. (a) Morphological characteristics of the liver, gut, and, swim bladder of the control larva and (b) treated larvae with 1.3 mg/L of PCA for 96 hours. (c) Histological section of the control larva with 144 hpf. (d) Normal hepatocyte morphology of the liver of control larvae and (e) (f) abnormal hepatocyte of the liver of the larvae treated with 1.3 mg/L of PCA

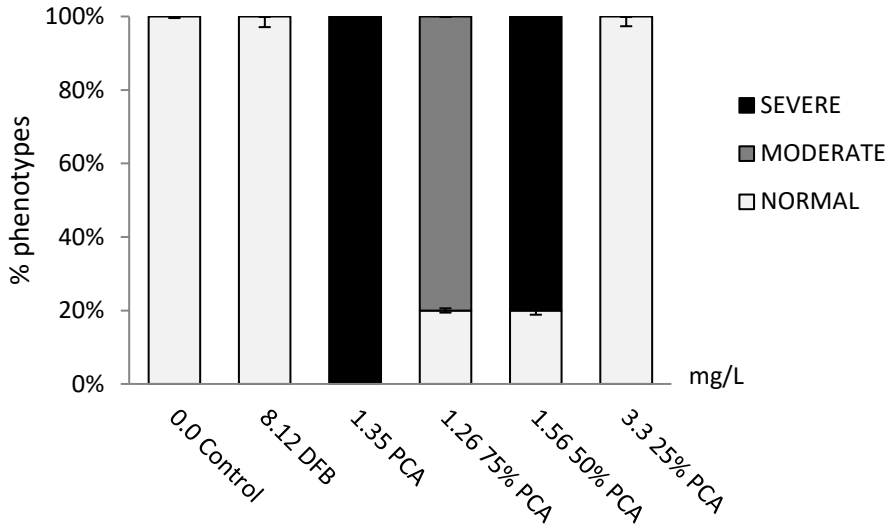

Fig. 5. Morphologic abnormalities induced by DFB, PCA and mixtures thereof after 96h of exposure larvae (144 hpf) zebrafish (n=10). Failure to inflate the swim bladder was scored as a moderate abnormality, and occurrence of one additional phenotype (i.e., amorphous liver, and dilated gut) was scored as severe. All experiments were repeated on three clutches, and error bars indicate standard deviation.

4. DISCUSSION

The knowledge of the toxic effects of mixtures is very important for the establishment of maximum permissible levels of the xenobiotics in the aquatic environment and there is a gap in the scientific literature on the environmental impact of the mixture of the DFB and PCA in the aquatic environment.

The acute toxicity tests showed that mixtures of DFB with PCA were more toxic to fish than insecticide itself. However, the mixtures were no more toxic than isolated p-chloroaniline. These results showed that the mixtures of DFB with PCA may influence the toxicity of the PCA in an antagonistic manner resulting less mortality of the organisms. The mixture with 75% of PCA caused more mortality of organisms among the mixtures evaluated, followed by mixtures with 50% and 25% PCA. However, concentrations of xenobiotics found in aquatic environmental usually do not occur at levels that result in direct mortality except in the case of accidental spills, e.g., PCA has been found in lakes and rivers at concentrations of 0.001 mg/L (Bresch et al., 1990; Hasenbein et al., 2015; Wegman and De Korte, 1981) and DFB is generally used in fish farming at random concentrations ranging from 0.5 to 2 $mg.L^{-1}$(He et al., 2013a; McGrath and Li, 2008; Vliegenthart et al., 2014) . Therefore, LC50 values obtained were used for calculations of sublethal concentrations of the compounds and mixtures thereof and showed that the larval phase of zebrafish was more sensitive to the compounds and

their mixtures than the adult fish. Similar results have been reported for larvae zebrafish exposed to other compounds (Cao et al., 2016; Mu et al., 2013; Wang et al., 2009, 2017).

Acute toxicity tests with DFB, generally are executed only with adult fish and there is an extensive database, which makes indispensable investigations that assess its toxicity during all the stages of the organism life cycle (S. A. Fischer and Hall, 1992; Jonsson et al., 2015; Pereira Maduenho and Martinez, 2008b). In addition, acute toxicity tests are determined with juvenile or adult fish that subjected to considerable pain and suffering which arouse ethical concern (Nagel, 2002).

The zebrafish embryo assays complemented the acute tests with adult fish exposed to mixtures and showed more informative. Hatching time has used as an endpoint in zebrafish embryos exposed to DFB, PCA and their mixtures. Hatching of zebrafish embryos from the chorion occurs within 48 - 72 hpf and involves a combination of biochemical and physical mechanisms (Kimmel et al., 1995; Li et al., 2017). The embryos exposed to mixtures of DFB and PCA presented a delay in hatching while the embryos exposed to the isolated compounds started to hatch with 72hpf. Hatching delay/failure of zebrafish embryos can occur due to different toxic mechanisms like inhibition of the enzyme chorionase and/or in the weakened spontaneous muscular movement of the larva to break the chorion and emerge (Hallare et al., 2004; Li et al., 2017). The embryos exposed to mixtures of DFB and PCA that had delayed hatching presented inability to break the chorion. Similar results were obtained for zebrafish embryos that were hatching retarded when prolonged exposed to 0.5 mg/L of 4-chloroaniline (Burkhardt-Holm et al., 1999c). The abnormal development also corroborates hatching delay (Li et al., 2017). Mixtures containing 75% and 50% PCA despite not causing mortality of embryos such as single PCA caused abnormalities in organisms during exposure. Embryos that were exposed to these mixtures for 96 hours and were able to develop and hatch had abnormalities such as pericardial edema, yolk sac edema, and, spine malformation. The mixture with 25% of PCA caused no mortality and no change in the development of the embryos as the other proportions of the mixtures and the isolated compounds. Therefore, presumed that in this proportion of the mixture the compounds interact an antagonistic manner. Many mixtures have antagonistic effects because one of the compounds induces a change in toxicokinetics, e.g., absorption and metabolism rates, etc (Hernández et al., 2013; Wang et al., 2017).

No studies were found in the literature to support the abnormalities verified during the embryonary development of zebrafish to DFB and its mixtures with PCA. However, a search with PCA in early life stages of zebrafish showed several similar abnormalities as spinal

deformation, the formation of edema in the trunk region, abnormal eye, trunk pigmentation, besides the lack of eye and mouth formation (Burkhardt-Holm et al., 1999c).

The mechanisms of embryo toxicity of compounds and its mixtures in zebrafish are not yet described, but pericardial edema can be associated with yolk sac lesion. Since the embryo uses endogenous yolk nutrients, yolk sac damage may obstruct nutrient supply during embryonic development causing abnormalities in the heart, such as pericardial edema, due to energy limitation (Kodde et al., 2007; Raldúa et al., 2008). Generally, pericardial edema occurs with yolk sac edema (Mu et al., 2013) as it was observed in embryos exposed to 46.8 mg/L of the mixture with 75% PCA (Fig. 2c). Our observation can be supported once pericardial edema may be associated with leaks in the endothelial vessels causing cardiovascular dysfunctions that promote the separation of this abnormality from the yolk sac edema (Hallare et al., 2005).

Spine deformations can be linked to depletion or deregulation of ions like calcium and phosphorus or with a reduction in myosin and myotonia, both required for normal embryonal development (Cheng et al., 2000; Hallare et al., 2005; Muramoto, 1981). Aberrations such as "Spina bifida" can be caused by teratogenic substances within the most sensitive developmental phase of ontogenesis in zebrafish embryos (Nagel, 2002). This abnormality and other spine deformations (scoliosis and lordosis) were observed in embryos/larvae exposed to 46.8 mg/Lof the mixture with 75% PCA (Fig. 2b).

Alteration of lipid synthesis and metabolism may cause yolk sac edema since the endogenous lipid reserves in fish eggs are in the form of yolk globules (Wiegand, 1996). Different methods have been used to assess the toxicity of xenobiotics in various organs of the transparent larvae of zebrafish and the gross and microscopic visualization of phenotypic endpoints has shown high-throughput screening (He et al., 2013a; McGrath and Li, 2008; Vliegenthart et al., 2014).

Zebrafish are closely related to humans and share many biological traits, genes, developmental processes, anatomy, physiology, and behavior (McGrath and Li, 2008; Parng et al., 2002). Therefore, we can infer that the compounds studied may cause similar effects, observed in the larvae, in humans.

The same phenotypes analyzed in this work were also observed in other studies utilizing zebrafish to assess toxic effects of various xenobiotics in mammalian. (Fehr et al., 2016; He et al., 2013b; Hill et al., 2012, 2005; McGrath and Li, 2008; Parng et al., 2002; Stinckens et al., 2016; Zhang et al., 2003).

Since DFB and PCA can direct or indirectly affect the environment and humans, the toxicity caused by sublethal doses of these compounds and their mixtures in embryos and zebrafish larvae is of great concern

5. CONCLUSIONS

Among the mixtures analyzed, 75% PCA displayed the highest toxicity, followed by 50% PCA, whereas 25% PCA exhibited the lowest toxicity to Zebrafish. The larval stage of zebrafish was the most sensitive and informative to compounds and mixture thereof. The presence of DFB decreased the toxic effects of PCA to the organisms and the mixture containing 25% of PCA showed antagonistic effects.

DFB, PCA, and their mixtures have deleterious effects on zebrafish adults and during early stages and may cause damage to the aquatic environment and to human health. The range of endpoints used on the embryo and larval test contributes to a better understanding of the toxicity of these compounds and their mixtures.

The procedures enabled assessment of a range of morphological changes with low cost, little time, space and waste generated.

This work reinforces the importance to study the joint action of compounds and fills, in part, a gap in the scientific literature regarding the environmental impact of DFB and its metabolite PCA on aquatic organisms.

CONCLUSÕES GERAIS

O inseticida diflubenzuron não foi tóxico para os peixes nos testes de toxicidade aguda. No entanto, concentrações subletais e mais próximas das que são utilizadas indevidamente na piscicultura foram capazes de provocar alterações a nível bioquímico e no desenvolvimento dos peixes. Adicionalmente, a mistura do inseticida com 75% de seu metabólito p-cloroanilina foi capaz de provocar efeitos tóxicos agudos e subletais maiores aos organismos do que os compostos individualmente. Contrariamente, o efeito antagonista foi observado na mistura do DFB com 25% de PCA. É difícil identificar com clareza os riscos das misturas desses compostos no meio ambiente. Neste sentido, a análise dos efeitos tóxicos das misturas do DFB e PCA foi importante, pois os dados obtidos podem delinear futuros estudos de biomonitoramento.

A presença do solo nos testes mimetizou o que pode ocorrer com os compostos no ambiente aquático. Em laboratório o solo diminuiu a biodisponibilidade dos compostos e de suas misturas na água devido a adsorção dos compostos às partículas dos solo. No entanto, uma vez ligados a essas partículas os compostos podem permanecer mais tempo no ambiente sem sofrer degradação e colocar em risco espécies sensíveis que habitam o solo.

Em relação aos organismos testes, tanto as tilápias quanto o zebrafish sofreram alterações fisiológicas significativas mediante a exposição às misturas. Apesar de não poder comparar a sensibilidade das tilápias com a do zebrafish, a utilização das duas espécies distintas de peixes foi essencial para investigação dos efeitos tóxicos das misturas no ambiente aquático, pois uma única espécie de organismo pode não representar totalmente os efeitos causados por xenobióticos em um determinado ecossistema. Os testes com os embriões e larvas de zebrafish foram mais informativos do que os testes que empregaram os peixes adultos, porém, o conjunto destes ensaios foi mais útil, pois forneceu dados qualitativos e quantitativos acerca dos compostos.

As enzimas extraídas das brânquias, primeiros órgãos a entrar em contato com os xenobióticos, responderam bem a exposição aos compostos e suas misturas, assim como as enzimas do fígado, órgão responsável pela metabolização dos xenobióticos. As enzimas avaliadas forneceram informações úteis sobre os efeitos adversos decorrentes da interação entre os organismos e as misturas dos compostos que podem ser de grande valor, seja para a tomada de decisões sustentáveis na piscicultura ou para avaliação de riscos.

Como o diflubenzuron e suas misturas com a PCA apresentou riscos aos organismos estudados, é necessário que haja divulgação dos resultados obtidos neste trabalho

em meios de comunicação acessíveis aos usuários destes compostos a fim de promover uma conscientização do seu uso.

Por fim, este trabalho preenche, em parte, uma lacuna existente na literatura científica a respeito do impacto ambiental em organismos aquáticos de um inseticida de uso frequente e não registrado na piscicultura, DFB, e de seu metabólito PCA, compostos que pouco se conhece e a intração entre eles e com o ambiente aquático.

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ANEXO

Anexo 1. Protocolo do comitê de ética no uso animal.

UNICAMP

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Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"TOXICIDADE COMPARATIVA DO DIFLUBENZURON E p-CLOROANILINA EM FOSFATASES E ENZIMAS ANTIOXIDANTES DE ORGANISMOS NÃO-ALVOS"** (protocolo nº **2756-1**), sob a responsabilidade de **Prof. Dr. Hiroshi Aoyama / Darlene Denise Dantzger**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **25 de junho de 2012**.

Campinas, 25 de junho de 2012.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/

Anexo 2. Declaração de direitos autorais

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Evaluation of the toxic effects of diflubenzuron, p-chloroaniline and mixtures thereof in tilapia (Oreochromis niloticus) and zebrafish (Danio rerio), in the presence and absence of soil**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 18 de outubro de 2017

Assinatura : _____
Nome do(a) autor(a): **Darlene Denise Dantzger**
RG n.° 32762605-7

Assinatura : _____
Nome do(a) orientador(a): **Hiroshi Aoyama**
RG n.° 2983293-7