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

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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 (*Oreochromis niloticus*) 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 *(Oreochromis niloticus)*. 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 (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). Thus, populations of cladoceran and copepods can be completely eliminated for extended periods of exposure to DFB concentrations above 7 µg/L (Fischer and Hall, 1992). Furthermore, diflubenzuron, when metabolized or degraded, can generate p-chloroaniline (PCA), a highly toxic chemical compound, known to induce splenic and bladder as well as liver tumors in mammals (Chhabra et al., 1991). 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).

The worrisome and complex scenario 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. 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 (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

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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 (Eltholth et al., 2015).

The evaluation of enzymes extracted from organs (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% autoclaved commercial peat (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)* was approved by the Ethics Committee for the Use of Animals of the State University of Campinas (CEUA/UNICAMP) under the registration N° 3641-1 (Law N° 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 exposure period of the fish to the isolated compounds and mixtures thereof were 96h during which the organisms were not fed. At the end of the exposure period of the fish, 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 biochemical analyses, 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 96hh, 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 96h, fish were collected, anesthetized with benzocaine (0.1 g/L) and sacrificed by spinal cord section, in accordance with the principles of the Ethics Committee for the Use of Animals of the State University of Campinas (CEUA/UNICAMP, Campinas, SP, Brazil) to avoid the suffering of organisms. This anesthesic has been used by authors of the present work in previous studies that evaluated enzymatic and hematological parameters (Sampaio et al., 2016), as well as by many others authors who evaluated enzymes activities like CAT (Modesto and Martinez, 2010), AlP (Signor et al., 2010), and AcP (Peruquetti et al., 2010). 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 10.000 × *g*, for 20 min, at 4 °C and the supernatant was collected. The supernatant was stored at − 80 °C and after used in the following biochemical analyses: activities of catalase (CAT), alkaline (AlP), and acid (AcP) phosphatases, alanine (ALT) and