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Combined toxicological effects of pesticides: A fish multi-biomarker approach

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ABSTRACT

The combined effects of two synthetic insecticides on a wide array of biomarkers in the freshwater fish *Piaractus mesopotamicus* were studied. Fish were exposed to sublethal concentrations of endosulfan (ED), lambda-cyhalothrin (LC), and the combination of both pesticides for 96 h. The set of analyzed biomarkers included morphometric and hematological parameters, transaminases and alkaline phosphatase activities, antioxidant enzymes activities and oxidative damage biomarkers measured in gills, liver, kidney, brain and muscle. According to the principal component analysis, the most significant effects were produced by the ED–LC combination. So, the mixture of both insecticides produced an increase in the liver-somatic index, hematological changes related to immunological biomarkers (increased white blood cells count, and alterations in the differential leukocytes count), decreased liver transaminases activity, antioxidant enzymes induction in almost every tissue, and lipid peroxidation levels increases in liver, kidney and brain of exposed fish. Our results suggest deleterious effects of ED and LC insecticides in combination, and support the usefulness of the multi-biomarker approach for the characterization of toxicological mechanisms induced by pesticides.

1. Introduction

Pesticides are widely used in agricultural and urban areas, and their mixtures are frequently used to improve efficacy and reduce cost of crop pest treatment. The methods employed for pesticide application (spraying and dusting) enable them to enter the aquatic ecosystem (Elezović et al., 1994). Thus, aquatic organisms are inevitably exposed to a multitude of toxicologically and structurally different pesticides. Although toxicity of individual pesticides has been extensively studied in fish (Joseph and Raj, 2011), the toxicological impact of their combinations is relatively less understood. So, more studies are necessary in order to understand their interactions on the living system and to evaluate their risk assessment.

The combined use of a set complementary biomarkers that can both signal exposure to contaminants and quantify their impact on living organisms, enables a more comprehensive and integrative assessment of biochemical and cellular effects induced by environmental pollutants (Linde-Arias et al., 2008; Cazenave et al., 2009). Consequently, the multi-biomarker approach has gained considerable interest in ecotoxicological research, and has been recently applied in both field and laboratory studies (He et al., 2012; Jolly et al., 2012; Matozzo et al., 2012; Liang et al., 2013).

Organochlorines and pyrethroid insecticides are among the most frequently used classes of pesticides, overlapping in soybean crops. In Argentina, Endosulfan (ED) is one of the remaining organochlorine pesticides registered and widely used for control of a large spectrum of insect pests (Miglioranza et al., 2003). ED concentrations from 0.2 to 13.5 $\mu g\,L^{-1}$ have been found on water bodies near rice fields in neotropical wetlands, and concentrations from 0.1 to 0.7 $\mu g\,L^{-1}$ on mountain rivers (Baudino et al., 2003; Silva et al., 2005). Similar to ED, the pyrethroid lambda-cyhalothrin (LC) is used in a wide range of crops. Although residues of other pyrethroids have been detected in water and sediment samples from streams and rivers in Argentina (Jergentz et al., 2005; Marino and Ronco, 2005), no data about environmental LC levels are available. The lack of information about this insecticide is likely related to its recent registration and current use (US EPA, 2001).

Due to its effects, both insecticides are considered highly toxic to fish and aquatic invertebrates (US EPA, 2002; Gu et al., 2007). It has been shown that individual ED and LC exposure can significantly induce antioxidant enzymes and oxidative stress (Ballesteros et al.,

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2009; Piner and Üner, 2012), and alter hematological parameters, transaminases, and alkaline phosfatase (ALP) activities in fish (Chandrasekar and Jayabalan, 1993; Hii et al., 2007; Girón-Pérez et al., 2008). In spite of this, no data on the effect of these insecticides together in aquatic organisms are available.

Piaractus mesopotamicus (Pisces, Characidae) is a South American omnivorous fish, widely used in fish farming due to their good consumer acceptance and high growth rate (Jomori et al., 2003). Thus, this study is aimed at evaluating multi-biomarker responses in *P. mesopotamicus*, after controlled exposure to commercial formulations of the organochlorine ED and the pyrethroid LC alone and in combination. The multi-biomarker approach proposed in the present study was focused simultaneously on morphometric parameters, antioxidant defenses, tissue damage, and hematological responses in fish.

2. Materials and methods

2.1. Fish

Juvenile *P. mesopotamicus* ($n = 160$; 8.1 ± 0.5 cm standard length; 23.9 ± 4.4 g) were obtained from a local fish farm. For acclimation purpose, fish were held in 150-L tanks containing well aerated dechlorinated water for two weeks, and fed once daily with dry commercial pellets. Fish feeding was suspended 24 h before the beginning of the tests.

2.2. Chemicals

ED (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-ethano-2,4,3-benzodioxathiepin-3-oxide) and LC [(RS)-alphacyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2,-dimethylcyclopropanecarboxylate] test solutions were prepared from commercial formulations containing 35% (Zebra Ciagro®, CAS No. 115-29-7, Ciagro Argentina S.A.) and 5% (Cilambda®, CAS No. RN [71751-41-2], Ciagro Argentina S.A.) active ingredient, respectively. Since technical-grade ED has xylene as vehicle that contribute to its water solubility, stock solution was prepared by the direct addition of technical-grade solution to 25 ml of ultrapure water. A stock solution of technical-grade LC was prepared dissolving it in 25 ml of acetone. Necessary volumes of stock solutions were added to the aquaria to achieve the desired final concentrations (solvent = 0.003% per aquaria). Stock solutions used in this study were always freshly prepared when needed.

2.3. Experimental design

Tests were conducted in 25-L glass aquaria under semi-static conditions. The experiments were carried out in 12:12 h light–dark cycles, and the test water conditions were: pH 6.9 ± 0.2, total hardness 48 ± 0.1 ppmCO_3Ca, and temperature $25 \pm 1\,^{\circ}C$. The aquarium solutions were renewed daily by transferring the fish to another aquarium. Experimental insecticide concentrations were calculated according to the active ingredient percentage present in the commercial formulation; and quantified at the beginning of each chemical renewal period by GC-ECD; showing recoveries >90% of the nominal value for both insecticides.

In order to determine insecticide sublethal concentrations, static 96-h acute toxicity tests were performed in accordance with OECD Guidelines for Testing of Chemicals (OECD, 1992). Fish ($n = 10$ per treatment) were individually exposed to five different nominal concentrations of ED (2.2, 2.8, 3.5, 4.4, and 5.5 $\mu g\,L^{-1}$) and LC (1.8, 2.2, 2.8, 3.5, and 4.4 $\mu g\,L^{-1}$). An additional group, which was kept in tap water, served as the control one. Mortality of test organisms was recorded when opercular movements stopped, and dead individuals were removed instantly. Accurate records of mortality counts were maintained at a regular interval of 12–96 h. LC_1 values were calculated for 96 h using US EPA probit software 1.5 free version (US EPA, 1992). At the end of the experiments, 96-h LC_1 values were calculated as 2.2 for ED and 1.4 $\mu g\,L^{-1}$ for LC.

In the sublethal toxicity test, 50% LC_1 values of both insecticides were applied. The selection of the 50% LC_1 was based on the methodology used in previous studies (Bacchetta et al., 2011a,b). Fish ($n = 10$ per treatment) were individually exposed to the following treatments: 0 (control), ED (1.1 $\mu g\,L^{-1}$), LC (0.7 $\mu g\,L^{-1}$) and a combination of both insecticides (1.1 μg ED L^{-1} + 0.7 μg LC L^{-1}, ED + LC), during 96 h. Fish were anesthetized and measured, weighted, sacrificed and dissected. Brain, gills, kidney, liver, and muscle were immediately frozen in liquid nitrogen and stored at −80 °C until biochemical determinations were carried out.

2.4. Biomarkers

2.4.1. Morphometric and hematological parameters

The condition factor (CF) and the liver somatic index (LSI), were calculated according to Goede and Barton (1990).

Blood was extracted from the caudal vessel by dissection of the caudal peduncle (Reichenbach-Klinke, 1980). Red blood cells (RBC) counts were performed with a Neubauer chamber and hematocrit (Ht) values were determined by the micromethod. Hemoglobin concentration (Hb) was measured by the cyanomethemoglobin method at wavelength of 546 nm (Houston, 1990). Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated from primary indices.

A drop of freshly collected blood was smeared on clean slides to estimate the total white blood cells (WBC) counts and for determination of leukocyte frequency according to Tavares-Dias and de Moraes (2007).

2.4.2. Transaminases and alkaline phosphatase

Samples of liver and kidney from each individual fish were homogenized in phosphate buffer (pH 7.4). The homogenate was centrifuged at 25,000 × *g* at 4 °C for 10 min, supernatant collected, and stored at −80 °C for enzymatic studies. Aspartate aminotransferase (AST) (L-aspartate-2-oxaloglutarate aminotransferase; EC 2.6.1.1) and alanine aminotransferase (ALT) (L-alanine-2-oxaloglutarate aminotransferase; EC 2.6.1.2) activities were estimated according to Reitman and Frankel (1957). Alkaline phosphatase (ALP) (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity was determined colorimetrically using a commercial kit (REF 1361003 Wiener Lab®, Argentina). Each sample was measured by triplicate and the enzymatic activity was calculated in terms of protein content (Bradford, 1976).

2.4.3. Antioxidant defenses

Enzyme extracts from each tissue (brain, gills, kidney, liver, and muscle) were prepared from each individual (not pooled). Briefly, tissues were homogenized using 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (DTE). The homogenate was centrifuged at 20,000 × *g* (4 °C) for 30 min, and the supernatant was collected and stored at −80 °C for enzyme measurement.

The activity of glutathione-*S*-transferase (GST, EC 2.5.1.18) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Glutathione reductase activity (GR, EC 1.6.4.2) was assayed according to Tanaka et al. (1994). The activity of glutathione peroxidase (GPx, EC 1.11.1.9) was determined according to Drotar et al. (1985), using H_2O_2 as substrate. Catalase activity (CAT, EC 1.11.1.6) was determined according to Beutler (1982). The enzymatic activity was calculated