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Effects of propanil on the European eel *Anguilla anguilla* and post-exposure recovery using selected biomarkers as effect criteria ☆, ☆☆

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ABSTRACT

The aim of this study was to assess the physiological response of *Anguilla anguilla* to propanil and the degree of recovery after being moved to clean water. Preliminary acute toxicity test was carried out in the laboratory and the median lethal concentration (LC50) at 96 h was calculated as 31.33 mg/L (29.60–33.59 mg/L). NOEC and LOEC values (at 96 h) were also calculated as 20 and 25 mg/L, respectively. The fish were exposed to 0.63 and 3.16 mg/L of propanil for 72 h and allowed to recover for 144 h. Total proteins (TPs), γ-glutamil transpeptidase (γ-GT), alanin aminotransferase (AlAT), alkaline phosphatase (AP), lactate dehydrogenase (LDH) and water content (WC) were assayed in muscle and liver tissues, liver somatic index (LSI) was also determined. Liver TPs and γ-GT activity decreased after propanil exposure while AlAT and LDH increased. Muscular AP, AlAT and proteins decreased in intoxicated eels while LDH and γ-GT activities increased. WC increased in both tissues after herbicide exposure as well as LSI. These results revealed that propanil affects the intermediary metabolism of *A. anguilla* and that the assayed enzymes can be used as good biomarkers of herbicide contamination. However a longer recovery period should be necessary to re-establish eel physiology. The parameters measured in the present study can be used as herbicide toxicity indicators and are recommended for environmental monitoring assessments.

1. Introduction

Propanil (3,4-dichloropropioanilide) is a selective contact herbicide which is used to control barnyard grass (*Echinochloa crusgalli*), broadleaf weeds and for post-emergent treatment of rice (*Oryza sativa*) (Moore and Farris, 1997). Its major metabolic pathway in microsomal incubations is acylamidase hydrolysis to 3,4-dichloroaniline (DCA) (McMillan et al., 1990). Propanil seems toxic to ictiological fauna, even at sublethal levels. Call et al. (1983, 1987) reported significantly lower eggs hatches, death and pericardial deformities in survival of the fish *Pimephales promelas* after sublethal exposure to propanil, joint to swollen bodies with reddish zones of haemorrhaging along with the visceral mass. Lipid peroxidation and oxidative stress had been cited in the freshwater fish *Carassius auratus* as consequence of the exposure to DCA, which could explain partially the propanil toxicity to fish (Li et al., 2002, 2003).

Changes in rice management practices have greatly reduced the concentration of pesticides in the runoff waters. However, during the spraying operations the concentrations of propanil, while not acutely toxic, deserve possible concern for their sublethal effects. Levels of 0.071 and 0.470 mg/L of propanil and DCA, respectively had been detected in paddy field from Eastern Spain 8 days after the end of treatments (Santos et al., 1998). It is important to understand the toxicity of herbicides on non-target aquatic organisms because of the large pesticide field application and the risk of their mixture in the aquatic environment (Moore et al., 1998).

European eel (*Anguilla anguilla*) is a fish of considerable economic importance in the European Community. Immature, feeding (yellow stage) eels occur in water reservoirs and connecting channels into areas with intensive agriculture where propanil is applied. It is essential to determine whether this herbicide affects the individuals of this species. This fish species contains large amount of fat, so pesticides will accumulate in eel tissues more than in other fish species (Holmberg et al., 1972). The European eel, especially during its depredator freshwater stage,

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☆☆ Formal institutional assurance: The University of Valencia (Spain) guarantees that all the experimental fish used in the present study were maintained in the laboratory following national and institutional guidelines for the protection of animal welfare in accordance with our Institutional Animal Care and Use Committee.

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yellow eel, seems to be a species highly exposed to pollutants (Larsson et al., 1991). Lokman et al. (2003) emphasized the importance of the normal growth and development of the yellow eel because of numerous changes associated with profound alterations in morphology and physiology of the animals in order to prepare them for the silver stage (sexual maturation).

The purpose of the present investigation was to study, under controlled laboratory conditions, the impact of short and continuous exposure of the European eel to sublethal propanil contaminated water on key enzymes involved in muscular and hepatic metabolism and detoxification routes used as biomarkers of herbicide poisoning. In addition, the propanil-exposed fish were allowed to recover for 6 days in clean water. Juvenile eels (commercial size) were used as test organism.

2. Material and methods

2.1. Test fish

Eels of the species, *A. anguilla*, were obtained from a fish farm. Yellow eels (weight, 20–30 g; length, 16–20 cm) were selected to minimize the effects of sex variation because at this development stage no sex differences are observed. Animals were acclimatized to laboratory conditions for one week in 300 L glass aerated water tanks before starting the experiments (Sancho et al., 2000). Eels did not respond to feeding attempts in the laboratory, but all animals were healthy. Mortality was observed neither during the acclimation period nor during experimental time.

2.2. Test system

The tanks were supplied with a continuous flow of aerated and dechlorinated tap water (temperature: $20\pm1\,^{\circ}C$; total hardness: 240 ± 10 mg/L as $CaCO_3$ according to the Merck classification, Aquamerck 8039, Germany; pH 7.9 ± 0.2 using a Crison pHmeter; alkalinity 4.0 ± 0.5 mmol/L, Aquamerck 11109, Germany). The light/dark period was 12/12 h (Sancho et al., 2000).

2.3. Chemicals

Ethyl 3-aminobenzoate, methanesulfonic acid salt 98% (MS222) was purchased from Aldrich Chemical Corporation Inc. (Milwaukee, WI, USA). All other reagents used in this study were obtained from Sigma Chemical (St. Louis, MO, USA). Kits for enzymatic assays were purchased from Spinreact S A (Spain).

The propanil used in the experiments was 80% pure (*IVIA*, *Valencia*, Spain). Stock solutions (50 and 10 mg/L for acute and subacute toxicity experiments, respectively) were prepared by dissolving propanil directly in experimental water immediately before to each experiment.

2.4. Toxicity testing protocol

Acute toxicity tests were carried out in our laboratory in order to calculate the 96h-LC_{50} for propanil in *A. anguilla*, based on OECD Guidelines (1998). In acute toxicity tests, groups of ten eels were placed in 45 L continuously aerated glass tanks and exposed to 20, 25, 30, 35, 45 and 50 mg/L of water for 96 h. Water characteristics as well as test system were already described above. The control eel group was kept in clean water as in the experimental sets. Mortality was recorded after 24, 48, 72 and 96 h, and LC_{50} values and its confidence limits (95%) were calculated by the Litchfield and Wilcoxon Method (1949). NOEC and LOEC values were also calculated after 96 h exposure. The test was carried out in triplicate and fish did not receive food during the experimental period, medium renewal was done every day. The subacute test concentrations used in this study ($1/50 = 0.63$ and $1/10 = 3.16$ mg/L) were based on these results.

Subacute propanil experiments were carried out in a continuous flow-through system based on OECD Guidelines (1998), in 300 L aerated glass aquaria. Eels were exposed to both propanil concentrations (0.63 and 3.16 mg/L) for 72 h and then a recovery period of 144 h in clean water was allowed. For the exposure period, the herbicide was previously dissolved in water making a 4 L stock solution and this solution was supplied to a glass mixing chamber with tap water which was connected to a perfusor pump (Gilbson, Minipulse 3) that generated a constant solution flow (2.44 mL/min). The outlet was connected to the 300 L aerated test aquarium. This diluted the pesticide to the desired concentration (0.63 or 3.16 mg/L) by a constant water flow. In this way, the aqueous test solution was renewed 3 times a day. This system was connected 24 h before the start of the experiments to reach a balanced propanil contaminated water in the test aquaria.

At 0, 2, 12, 24, 48, 56 and 72 h six eels were removed, rinsed with tap water and anaesthetized with MS222 at a concentration of 100 mg/L (Van Waarde et al., 1983). Animals were weighted and dissected out quickly on an ice-cold glass plate to remove liver and skeletal muscle samples, which were wet weighted and frozen at $-80\,^{\circ}C$ until analysis.

Gas chromatography analysis confirmed the presence of propanil in the water at the desired concentration over the entire exposure period (Santos et al., 2000).

In a second part of the experiment, eels previously exposed to both propanil concentrations during 72 h were transferred to clean water in a 300 L glass aquarium with the same flow-through system under the above described conditions but without herbicide (recovery period). Six eels were removed after 8, 24, 48, 96 and 144 h. The same tissues were taken out and stored at $-80\,^{\circ}C$ until analyses for its biochemical parameters determination.

At the same time, a control experiment was performed to test if the acclimatization of the animals to our laboratory conditions or the handling of the eels had any effect on the tested parameters. Therefore, eels were exposed to the same experimental aerated tap water under the same condition described above but without propanil. At the same exposure and recovery times, animals were removed and eel tissues dissected for metabolic determination.

2.5. Water content (WC) and liver somatic index (LSI)

One portion of both muscle tissue and hepatic organ from every individual fish was wet weighted and then dried for 72 h at $105\,^{\circ}C$ until constant weight and then reweighed to determine WC (Heath, 1984; De la Torre et al., 1999; Sancho et al., 2003).

LSI from every individual eel was calculated as percentage of wet weight organ (g) per wet weight (Kg) of the individual (Heath, 1995; Sancho et al., 2003).

2.6. Enzyme assays

Samples of liver and muscle tissue from each individual fish were homogenized with five volumes of 0.1 M phosphate buffer (pH de 7.2 at $0\,^{\circ}C$). The homogenates were centrifuged at 20,000 g ($4\,^{\circ}C$) for 10 min and the resulting supernatants were diluted five-fold with phosphate buffer (pH 7.4) and assayed rapidly for enzymatic determination.

Determinations were performed according to automated spectrophotometric methods provided by Spinrect (Spain) using a Chemistry Profile Analyzer (C.P.A.; Coulter Scientific, Margency, Francia). Final temperature of measurements was $37\,^{\circ}C$, corresponding to the incubation CPA temperature. Alanin aminotransferase activity (AlAT, EC 2.6.1.2.) was assayed by the method by Bergmeyer et al. (1978). The rate of NADH depletion was determined photometrically (340 nm) as the catalytic rate concentration of AlAT in the analyzed sample. The method of Wenger (1984) was used to measure the alkaline phosphatase (AP, EC 3.1.3.1.) activity. The rate of p-Nitrofenilphosphate hydrolysed to form p-Nitrofenol was monitored photometrically at 405 nm. γ-Glutamil transpeptidase (γ-GT, EC 2.3.2.2.) was analyzed using the method of Szasz (1969). The rate of the substrate L-γ-glutamyl-3-carboxi-*p*-nitroanilide cleavage to form 5-amine-nitrobenzoic acid was monitored at 405 nm. Finally, lactate-dehydrogenase (LDH, EC 1.1.1.27) was assayed according to Vassault (1983) method. The depletion rate of NADH as substrate was performed at 340 nm, every 30 s for 3 min. Enzyme activities were expressed as total activities in crude homogenate (μmol/min/g).

2.7. Total proteins (TP)

TPs content in the selected tissues, was estimated by applying the kit protein assay from Sigma Diagnostics® based on the method of Lowry et al. (1951) to each sample of the tissue homogenates after centrifugation (20,000 g, $4\,^{\circ}C$, 10 min). To the supernatant, an equal volume of trichloroacetic acid was added to precipitate the soluble proteins and centrifuged (3500 rpm, 10 min). The supernatant was discharged and the pellet was dissolved in Lowry reagent solution (1 mL). The tubes were kept at room temperature for 20 min. Then 0.5 mL of Folin-phenol reagent was added, and the colour after 30 min was reared at 750 nm in a spectrophotometer against a reagent blank. Bovine serum albumin was used as standard. Final concentration of TPs was expressed as milligram per gram of wet weight.

2.8. Statistical analysis

Individual eels exposed to sublethal propanil concentrations were grouped according to exposure and recovery times. Variables (no transformed data) were tested for normality (Kolmogorov–Smirnov test with Lilliefors significance correction) and variance homogeneity (Levene test). Mean values and standard deviations were calculated for each test group based on the values obtained for each individual tissue from six fish. The results were compared to determine treatment toxic effects by analysis of variance (ANOVA) and Duncan's significant difference test. Tukey's honestly significant difference (HSD) test was used to