Pesticide Biochemistry and Physiology 129 (2016) 63–69

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/ypest

Oxidative stress related to chlorpyrifos exposure in rainbow trout: Acute and medium term effects on genetic biomarkers

A. Benedetto [a,*], P. Brizio [a], S. Squadrone [a], T. Scanzio [a], M. Righetti [a], L. Gasco [b], M. Prearo [a], M.C. Abete [a]

[a] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, via Bologna 148, 10154 Torino, Italy
[b] Dipartimento di Scienze Zootecniche Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Via Leonardo da Vinci 44, 10095 Grugliasco, Italy

ARTICLE INFO

Article history:
Received 23 March 2015
Received in revised form 21 October 2015
Accepted 21 October 2015
Available online 23 October 2015

Keywords:
Chlorpyrifos
Chlorpyrifos oxon
Rainbow trout
Oxidative stress
Gene expression
Mass spectrometry

ABSTRACT

Organophosphates (OPs) are derivatives of phosphoric acid widely used in agriculture as pesticides. Chlorpyrifos (CPF) is an OP that is extremely toxic to aquatic organisms. Rainbow trout (*Oncorhynchus mykiss*) is considered as a sentinel model species for ecotoxicology assessment in freshwater ecosystems. An exposure study was carried out on rainbow trout to investigate genetic responses to CPF-induced oxidative stress by Real-Time PCR, and to determine the accumulation dynamics of CPF and toxic metabolite chlorpyrifos-oxon (CPF-ox) in edible parts, by HPLC–MS/MS. Among the genes considered to be related to oxidative stress, a significant increase in HSP70 mRNA levels was observed in liver samples up to 14 days after CPF exposure (0.05 mg/L). CPF concentrations in muscle samples reach mean values of 285.25 ng/g within 96 hours of exposure, while CPF-ox concentrations were always under the limit of quantification (LOQ) of the applied method. Our findings lead us to consider HSP70 as a suitable genetic marker in rainbow trout for acute and medium-term monitoring of CPF exposure, complementary to analytical determinations.

1. Introduction

Chlorpyrifos (CPF) is an organophosphate (OP) insecticide widely used to control a large variety of pests (for example, Coleoptera, Diptera, Homoptera and Lepidoptera families) in agricultural and animal farms [1]. Systemic and unselective cholinesterase inhibition by CPF, together with many known side effects, including endocrine disruption in early development and growth stages of vertebrates [2], are increasingly threatening the health of humans and several other animal species including freshwater fauna [3]. Imprudent agriculture practices and irrigation water are the main sources that result in the spread of these contaminants into the environment, and are responsible for water quality decline. Indeed, surveys performed in many countries have shown that CPF, together with other OP, triazine and pyrethroid insecticides are often reported as contaminants of surface and ground waters [4,5].

Seasonal use of OP pesticides and their short half-life often reduce the possibility of fully characterizing contamination dynamics and environmental fate; for these reasons their ecotoxicology assessments on aquatic species are becoming increasingly important [6].

In addition to cholinesterase inhibition, the main effects of these compounds and their metabolites are related to deregulation of pro-/anti-inflammatory cytokines [7,8] and oxidative stress [9,10]. Organophosphates like CPF could indeed promote in non-target species the activation of TNF-α release with induction of NF-kB [11], often related to heparanase and HSP70 alterations in early stages of inflammatory response [12]; in HaCaT cell lines CPF could induce the pyroptosis/apoptosis promoted by NLRP3 inflammasome [13]. Other main effects of CPF exposure concerned the oxidative stress, that emerges when the balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants and/or the accumulation of reactive oxygen species (ROS). Mobilization of anti-oxidative enzymes, with the scope of detoxifying ROS, is the main mechanism of cell defense, based on activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), glutathione S-transferase (GST), the thioredoxin system and other scavengers such as reduced glutathione (GSH) and related glutathione reductase (GSSG-R).

Therefore, the study of these oxidative stress-related biomarkers could represent a suitable tool for directly monitoring animal health and indirect monitoring of the environmental quality [14].

In this context, the aim of our study was to investigate, at the gene expression level, the responses and correlations of the anti-oxidative system to CPF exposure in the liver of rainbow trout (*Oncorhynchus mykiss*). This was chosen as a suitable fish model due to its high sensitivity to the effects of CPF, related to high catalytic rates mediated by CYP450 bioactivation [15]. The gene expression study was focused on liver-oxidative stress related enzymes, being liver primarily responsible for the metabolism of toxic substances, including CPF, as shown in other teleost fish [16]. In addition, chemical monitoring of CPF and oxon metabolites in water samples and muscle samples from exposed fish

* Corresponding author.
E-mail address: alessandro.benedetto@izsto.it (A. Benedetto).

http://dx.doi.org/10.1016/j.pestbp.2015.10.019
0048-3575/

could allow the acquisition of further information and possible correlations to the response of specific biomarkers.

Starting from the available data [17], an exposure protocol based on CPF LC_{50} for rainbow trout (0.05 mg/L) was performed to monitor acute toxicity responses followed by a detoxifying study to evaluate any possible long-term correlation between CPF contact exposure and the oxidative stress response.

2. Materials and methods

2.1. Animals and exposure protocols

A 96-hour treatment was arranged under semi-static conditions [18] at the Experimental Center of the University of Turin (Carmagnola, TO, Italy). A total of 170 female specimens of rainbow trout (average weight 193 ± 25 g, average length 27.5 ± 1.0 cm) were subdivided by random assignment to four 1200-liter indoor fiberglass tanks (A = 50 trout, B = 50, C = 30, and D = 40) supplied with running well water (average water temperature 13.6 °C, average dissolved oxygen 8.74 mg/L). Fish were hand-fed twice a day, 6 days a week to apparent satiation with a commercial diet (Optiline, Skretting). Feeding was stopped at 3 days before treatment and during the 96-hour CPF exposure.

CPF (60 mg) was solubilized in 20 ml 96% ethanol (*v*/*v*) and added to tanks C and D. Tank B was treated with solvent (20 ml ethanol alone), while tank A was left untreated.

Every 24 h, tank cleaning and solution restoration was carried out in all the fiberglass tanks, including tank A where there was no chemical exposure. During these procedures, all the specimens from each tank were temporary transferred into separate 500-liter plastic sinks; all the tanks were cleaned and then 0.05 mg/L fresh CPF solutions were added to tanks C and D (20 ml 96% ethanol was added to tank B). A total of 16 water samples were collected in clean 50 ml plastic tubes: eight samples prior to the daily maintenance procedures and eight samples at 1 h after restoring the exposure conditions in tanks B, C and D.

At the beginning of the treatment and subsequently every 24 h, five fish from tanks A, B, and D were randomly sampled (a total of 15 trout for each 24-hour sampling) and then euthanized with a lethal dose of MS222 (Sigma-Aldrich®), as shown in Table 1.

To monitor medium-term effects of CPF, at the end of the 96-hour acute exposure protocol, the remaining animals were kept in their respective tanks, feeding was recommenced and the same sampling procedures were applied every 7 days, for a period of 28 days (five specimens randomly sampled from each tank, excluding tank C).

Separate samples of liver and muscle fillets were then taken for genetic and chemical analyses.

Tank C was purely for mortality estimation and behavioral observations of rainbow trout exposed to 0.05 mg/L of CPF; for this reason no sampling was carried out, but dead animals were removed during the 24-hour cleaning/monitoring procedures (see Table 1).

Experimental design and animal handling procedures were approved by the institute Ethics Commission.

2.2. Condition index

During sampling procedures, the length and weight of collected specimens were noted, in order to estimate the Fulton's K factor, a known index used to quantitatively compare the condition of individual fish within a population, individual fish from different populations, and two or more populations from different localities or water conditions [19].

2.3. Chemical analysis

Fish tissues were extracted using the slightly modified Hassan et al. [20] method: 5 g of minced muscle was accurately weighed into a polypropylene tube and 25 μL Diazinon (used as an internal standard at a final concentration of 50 ng/g) and 5 mL of methanol were then added. Samples were sonicated in an ultrasonic bath at 40 °C for 15 min; tubes were then vigorously shaken and centrifuged at 3800 rpm for 5 min. Supernatants were transferred into clean glass tubes and an additional 5 mL methanol was added to the initial sample; the extraction procedure was repeated and the new extract was combined to the first one. A volume of 2 mL hexane was then added to the extract and tubes were vigorously shaken again for 5 min. After this step, 5 mL ultrapure water was added to the tubes to allow separation of the solvents. After centrifugation for 5 min at 3000 rpm, the hexane phase was evaporated under a nitrogen stream at 40 °C.

Water samples (5 mL) were extracted after addition of the internal standard and hexane; in this case as well, the organic layer was transferred and evaporated under a nitrogen stream at 40 °C.

Both residues were then dissolved in a mixture of 50 mL of acetonitrile (65%) and formic acid 0.1% *w*/*v* (35%).

The HPLC system consisted of an Agilent 1100 series (G1311A quaternary pump), a SecurityGuard C18 (4 × 3.0 mm ID) and a Synergi 4 μm MAX-RP 150 × 2 mm column, both supplied by Phenomenex, California (USA). An API 4000 Triple Quadrupole (ABSciex, Massachusetts, USA) was chosen as Mass Spectrometer.

Chromatographic separation was performed using acetonitrile (eluent A) and formic acid 0.1% *w*/*v* (eluent B) as mobile phases, at a flow rate of 0.5 ml/min: 65% solvent A at time 0, 72% solvent A at time 2.5 min, 100% solvent A at time 4 min and for 1 min. A total of 4 min were spent to re-equilibrate the column before the following run, for a total time of 9 min for each sample. A 10 μL-volume injection was applied in each run.

Electrospray ionization in positive mode was selected during mass spectrometry analysis; detection of two product ion transitions was monitored.

Table 1
Sampling program and specimen distribution in fiberglass tanks A, B, C and D. Five specimens were removed at each time point during both exposure and detoxification phases. Specimens in Tank C were not sampled, in order to estimate mortality rates.

Sampling program		Tank A	Tank B	Tank C (no sampling)	Tank D
	Time point	Negative group	Solvent only control group	CPF 0.05 μg/L exposed (mortality calculation)	CPF 0.05 μg/L exposed
Exposure	0 h (start)	50	50	30	40
	24 h	45	45	30	35
	48 h	40	40	28	30
	72 h	35	35	25	25
	96 h	30	30	22	20
Detoxification	7 days	25	25	22	15
	14 days	20	20	22	10
	21 days	15	15	22	5
	28 days (end)	10	10	22	0