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

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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] and [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] and [8] and oxidative stress [9] and [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 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 LC50 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.

Chlorpyrifos (CPF) and/or Chlorpyrifos-oxon (CPF-ox) were quantified comparing analyte/IS area ratio in the sample with the calibration curve, created with blank samples calibrated at suitable concentrations.

Limit of quantification (LOQ) of both analytes was 10 ng/mL in water and 15 ng/g in muscle.

2.4. Gene expression analysis

2.4.1. Primer design

All novel primer sets were designed using Primer express 3.0 software (Applied Biosystems). Primer concentrations for housekeeping genes were optimized for each assay by analyzing fluorescence signals on positive and negative control samples with serial primer dilutions, and selecting concentrations that

resulted in the highest fluorescence signal at the lowest Ct number with the expected melting curve profile. Primer sequences, optimal concentrations and amplicon lengths are reported in Table 2.

2.4.2. RNA isolation and reverse transcription

Total RNA was isolated from liver samples of all groups previously stored in RNA-Later solution (Ambion, Austin, TX, USA). RNA extraction was performed using Qiagen RNeasy Plus MiniKit (Qiagen, Basel, Switzerland) according to the manufacturer's protocol. Extracted RNA was treated with Turbo DNAse kit (Ambion) to avoid any traces of genomic DNA. Extracted RNA from all samples was quantified by fluorimetric analyses using Q-bit HS-RNA assay (Invitrogen, Carlsbad, CA, USA). A total of 1 μg of RNA from each sample was then transcribed to cDNA using the QuantiTect Reverse transcription kit (Qiagen).

2.4.3. Real time amplification

Real time PCR amplification was performed on an Applied Biosystems Step One Plus analyzer with Fast SYBR Green PCR master mix (Applied Biosystems). The reaction mix included 10 μL of SYBR Green 2 × master mix, a final concentration of each pair of primers as reported in Table 2; 2 μL of cDNA template and nuclease-free water to reach a final reaction volume of 20 μL.

The run method was set with a starting holding stage at 95 °C for 20 s, 40 cycles of amplification carried out with denaturation at 95 °C for 3 s, annealing and elongation at 60 °C for 30 s, followed by melting curve analysis.

Preliminary analysis for validation of a suitable housekeeping panel was performed as reported by Benedetto et al. [21].

Expression levels of the selected genes were determined by the ΔΔCT method [22] vs. two housekeeping genes, EF1-α and HPRT. Analysis was focused on genes established to be involved in oxidative stress, such as Heat Shock Proteins (HSP70, HSP90), SOD, GSSG-R, GSH-Px, Thioredoxin, Thioredoxin Reductase and GST [23]. We also tested serum albumin, a marker of hepatic activity and a known carrier of OP. Data were registered every 24 h for acute exposure, and every 7 days for the subsequent detoxification phase (Table 1).

2.5. Statistical analysis

ΔΔCT calculation and statistical analysis for measuring the significance of differences in gene expression profiles between treated and untreated groups was assessed by one-way ANOVA, corrected for multiple comparison by Tukey's test (results were considered significant at P values < 0.05), performed using the Expression Suite 1.0, Data Assist 3.0 (Applied Biosystems) and GraphPad Prism 6.0 software.

3. Results

3.1. Fulton's K indices, behavioral observation and mortality

The effects of pesticide in rainbow trout caused a significant reduction of Fulton's K indices in different CPF exposure groups (Fig. 1), with a reduction to 0.86 ± 0.03 mg/mm in the 72-hour exposed group, 0.83 ± 0.01 mg/mm in the 96-hour exposed group and 0.85 ± 0.01 mg/mm in the 7 days after CPF exposure group.

At the end of the 96-hour treatment, eight specimens died in tanks C (mortality = 23.3%). In the subsequent detoxification period no other deceases were recorded.

After 96-h of CPF exposure, specimens in tanks C and D showed a significant increase of behavioral alterations, typical for OP intoxication, such as lethargy and temporary muscular paralysis, followed by temporary loss of swimming balance (data not shown).

3.2. Chemical analysis

Concentration profiles of CPF in muscle samples within 96 h of exposure are shown in Fig. 2, together with concentration trends of CPF and CPF-ox in water. Concentrations of CPF and CPF-ox in water (two independent measurements from water samples collected from tanks C and D at the same time point) and mean CPF concentrations in muscle samples (from five specimens taken at each time point in tank D) are reported in Table 3.

CPF and CPF-ox were not detected in any muscle and water sample collected from untreated or solvent-treated control groups (tanks A and B), (i.e. < LOQ value).

During the 96-hour CPF exposure (0.05 mg/L, or rather 50 μg/L), CPF accumulation dynamics in muscle tissue of sampled specimens were described (Fig. 2), with concentrations shifting from 18.74 ± 7.52 ng/g, in the 24-hour exposed group, up to 285.25 ± 34.61 ng/g, recorded in the 96-hour exposed group, while CPF-ox concentrations in the same samples always resulted as being lower than the LOQ value.

At the same time, CPF and CPF-ox concentrations dynamics in water samples from tanks C and D, collected every 24 h during maintenance and fish sampling procedures, were recorded: CPF levels in the water ranged from unquantifiable concentrations (< LOQ) in all samples taken before cleaning procedures (at the end of every 24 h) to mean values of 26.43 ± 9.21 μg/L, recorded in samples collected 1 h after restoring exposure conditions.

3.3. Gene expression

Gene expression profiles on selected oxidative stress-related genes, recorded in the acute exposure protocol described in Section 2.1, are reported in Fig. 3; data recorded in the subsequent 28 days for the same targeted genes are shown in Fig. 4.

In the acute phase exposure, the main up-regulated gene was HSP70 in all groups exposed to CPF (24, 48 and 96 h) when compared with both the negative control group and the solvent group ($P < 0.05$). An additional 2-fold up-regulation of HSP70 expression was recorded at 14 days after acute exposure (Fig. 4).

Furthermore, GSH-Px expression was strongly increased during CPF exposure, especially in the groups where samples were collected at 72-h and 96-h after exposure compared with the negative control group ($P < 0.05$). However, observed alterations in the 72-hour and 96-hour exposed groups were not statistically significant when compared with the solvent group, in which GSH-Px expression was increased more than 3-fold, compared to the negative control group ($P < 0.05$).

The GSSG-R expression slightly increased in CPF-exposed groups (24-h, 48-h and 72-h), reaching a 2.5-fold induction in the 96-hour group ($P < 0.05$), as shown in Fig. 3, then returning to basal expression levels, comparable to both negative and solvent groups, in the subsequent 28 days of detoxification.

Liver serum albumin expression levels were also altered by CPF exposure, with progressive reduction of its expression in liver samples from CPF-exposed groups, reaching a significant halving in the 72-hour CPF-exposed group compared with the unexposed group ($P < 0.05$), which was not statistically significant when compared with the solvent group ($P = 0.11$).

4. Discussion

Many authors have reported that K index values may be greatly influenced by the stage of development of the reproductive organs, often unsuitable for toxicological assessments in unsexed populations [24], [25] and [26]. Therefore, when comparing K values, it is important to sample the individuals or populations at the same time of the year so that they are at the same stage of the reproductive cycle [27]. In our experimental conditions, the observed variations in Fulton's K mean values (Fig. 1) in different treated and untreated groups were, however, related exclusively to CPF exposure, when taking into account all requirements for specimen homogeneity and sample size (see Section 2.1). K mean values recorded in tanks C and D (fish exposed to CPF) were significantly lower than K values recorded in tanks A and B, up to day 14 after pesticide exposure, when Fulton's K index mean values in exposed fish also became similar to those reported in unexposed fish (Fig. 1).

Our findings confirm the general usefulness of condition indices, allowing a preliminary and indirect straight-forward evaluation of exposure effects, before proceeding with more expensive and time-consuming investigations.

The observed mortality in tank C was much lower than would have been expected from applying LC_{50} doses reported by previous toxicity studies [17] and [28], partially confirmed by mean CPF concentrations found in water samples (26.43 ± 9.21 µg/L), also lower than expected. Moreover, monitoring CPF and CPF-ox levels in the water medium during the exposure revealed periodic fluctuations in their concentrations (Fig. 2), requiring exposure conditions to be restored every 24 h. Our data confirm the low water solubility and the strong OP absorption by organic matter reported by Pehkonen and collaborators [29]. Moreover, when the solubility and stability of CPF in large volumes of water is increased by means of organic solvents such as methanol/ethanol [30], the half-lives of the pesticide could be however affected, to a lesser or greater extent, by several events, namely: shift in temperature range, acidification of water media (for metabolic activity of exposed animals, microbial growth, etc.), which can promote precipitation, degradation of CPF and photo-oxidation to CPF-ox and/or inactive metabolite 3,5,6-trichloro-2-pyridinol (TCP) [31]. Indeed, CPF-ox in water samples only increased to quantifiable levels at the end of every 24 h of exposure, before tank maintenance (see Table 3). These findings confirmed an intrinsically lower environmental stability of CPF and other OP pesticides, especially when compared with the previous generation of highly-persistent organochloride pesticides. Analytical determinations in muscle samples of CPF-exposed specimens indicated the progressive and significant accumulation of CPF in 96 h (up to 285.25 ± 34.61 ng/g), while CPF-ox concentrations in the same muscle samples were always under the LOQ. The high SD of CPF concentrations found in muscle samples (Table 3) highlights the inter-individual differences in CPF accumulation between specimens and the limits of exposure conditions applied, when grouping fish in high capacity fiberglass tanks is required instead of single and separate laboratory tanks for each specimen. However, these preliminary results confirm pharmacokinetics studies performed in other fish species [32]. Moreover, recent studies in mice and humans [33] and [34] revealed the reduced availability of CPF-ox in tissue, due to its high reactivity with serine, tyrosine and lysine residues of plasma proteins [35]; this feature could bring to more stable CPF-ox-proteins adducts in blood/plasma samples [36], limiting the detection of CPF-ox in its freeform [37].

Regarding biomarker profiling, preliminary data has reported that OP pesticides, such as CPF, could compromise the mechanisms of the cells to counteract oxidative stress and thus result in the generation of free radicals or free oxygen radical-scavenging enzyme systems [38]. However, analyses carried out in our study underlined that not only CPF, but also ethanol, could influence the expression levels of some oxidative stress-related markers. These alterations were particularly evident for GSH-Px (more than 3-fold induction in the solvent group compared to the untreated group), but less marked for albumin, SOD, GSSG-R and GST expression.

For example, albumin expression in livers of CPF-exposed fish was in fact reduced when compared with the unexposed control group (halved within 72 h of OP exposure); the

contribution of ethanol to alterations in mRNA transcription levels became evident when comparing the negative control group with the solvent-only exposed group.

Conversely, transcriptional induction in thioredoxin and thioredoxin reductase mRNA levels in both CPF and ethanol-exposed groups was observed within 96 h. Reported data by Pacitti et al., [14], highlight how the thioredoxin system (one of the main intracellular redox systems, implicated in the regulation of ROS accumulation) is rapidly involved in aspecific early responses to different oxidative stress events, ranging from toxic compound exposure to pathogen-associated molecules (PAMPs).

Distinguishing clear additive or opposing effects between CPF and the carrier solvent was not straight-forward; high standard deviations in mRNA levels of some of the considered biomarkers demonstrated the known effects of inter-individual biological differences [39], even when suitable actions were taken to limit confounding variance (for example, appropriate design of gene expression assays, normalization of biomarker expression against stable housekeeping genes, only strictly necessary handling of fish and proper stocking density to limit induced stress, sufficient number of biological and technical replicates, homogeneous groups in terms of sex, age, size, etc.).

The only considered target that resulted as being less affected by listed background variability, in our experimental conditions, was HSP70, for which a stable and persistently-induced expression in the livers of rainbow trout was reported up to 14 days after 96-h CPF (0.05 mg/L) exposure (Fig. 3 and Fig. 4).

The other Heat Shock Protein considered in present study (HSP90) did not shown significant up/down regulation caused by CPF exposure (Fig. 3 and Fig. 4): the reasons of such differential expression seemed not clear; previous studies have shown how HSP90 mRNA expression has been related to different types of stress events [40], such as in cortisol-induced transcriptional regulation of cytosolic glucocorticoid receptor [41], or in the modulation of AhR signaling and linked CYP450 gene battery in trout hepatocytes [42]. On the contrary, in a recent study on human liver cell lines stimulated with low doses of different pesticide mixtures, HSP90 mRNA level shown no basal alteration and was then chosen by the authors for normalization of raw data together with other housekeeping genes [43]. On the other hand, several reports have demonstrated that HSP70 exerts an anti-apoptotic effect, acting as a molecular chaperone in antioxidant and immune responses: the HSP family (including HSP70 and HSP90) therefore has different key roles to maintain protein homeostasis in the cellular survival pathways of different fish tissues [44], [45] and [46]. A prevalent involvement of HSP70 instead of HSP90 in pesticide exposure has indeed been reported in other fish species; a recent *in vivo* study reported significant upregulation in the expression of carp HSP70 and 70-kDa heat shock cognate protein (HSC70) with Atrazine and CPF treatment alone, or in combination [47].

In conclusion, our findings allow consideration of HSP70 as a suitable genetic marker for acute and medium-term monitoring of CPF exposure in rainbow trout, complementary to more expensive analytical determinations.

Chemical determination alone could indeed result as being inconclusive when only performed in environment samples (water, soil, etc.) due to the intrinsically short half-life of OP, but could became more significant when performed on fish muscle samples, where the rapid accumulation during exposure ensures detectable levels.

The synergic and single effects of CPF and chosen carrier on redox system perturbations, as well as the exposure conditions, resulted, in this study, as being less marked compared to those described by other authors (mortality observed was lower than expected from applying LC_{50} concentrations), will be investigated in future studies.

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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

Table 2
Sequences, optimal concentrations and amplicon lengths of gene expression targets. Elongation factor 1α (EF 1α) and Hypoxanthine phosphoribosyl transferase (HPRT) were adopted as housekeeping genes for normalization of $\Delta\Delta_{CT}$ calculations.

Target	Primer forward (5′-3′)	Primer reverse (5′-3′)	Optimal concentration [nM]	Amplicon length [bp]	References and/or NCBI accession
EF 1α	TCTGCCCCTCCAGGATGTC	TGGTGACATTAGCGGGGG	200/200	123 bp	Benedetto et al., (2011)
HPRT	GGCTACACACCAGACTTCA TAGGA	GAAGTACTCGTTGTAGTCTA GCGCATAT	250/250	61 bp	Benedetto et al., (2011)
Cu,ZN-SOD	GGACCGTATTCTTTGAGCAGGA	GCRTGGACGTGGAAGCCA	300/300	100 bp	AF469663.1
GSSG-R	CACCAGTGATGGCTTTTTT	ATATCCGGCCCCCACTATG	300/300	67 bp	Farmen et al., (2010)
Thioredoxin	ACCGTGCAGCCTAGAATGCT	GTGATGTCTCTCTTTGCAGTTCCTT	300/300	76 bp	Farmen et al., (2010)
Thioredoxin-Reductase	TCACCAGCGACGACCTGTT	TGGAGCGTACCATGACTGTCA	200/200	135 bp	HF969247.1 HF969246.1
GSH_Px	CCGACATCAGGAGAACTGCAA	CATTCACATCCACTTTCTCAAGGA	200/200	113 bp	NM_001124525.1
HSP70	GGGCCAGAAGGTGTCCAAT	CATTCAGCCCAGCGATCACT	250/200	101 bp	AB062281.1
HSP90	GCTCATGAAGGAGATCCTGGAC	AGTTGTCCCTCAGGGCCTG	200/200	149 bp	NM_001124591.1 NM_001124231.1
GST-α	CCTGGCTGCTGAAATCAAACT	GACAATAACGTCAGCCAATGAGAA	200/200	100 bp	NM_001160559.1
Ser Albumin	GAGATGTGGCCGACCTGTTC	CTCTCAGCGGCAGTCTTCTCA	200/200	100 bp	FN811665.1

Table 3
Chlorpyrifos (CPF) concentrations in muscle samples (mean of five determinations, one from each sampled specimen, collected from CPF exposed group). CPF and Chlorpyrifos-oxon (CPF-ox) concentrations in water samples collected from tank C and D.

Time point		Muscle samples	Tank	Water samples	
		CPF (ng/g)		CPF (μg/L)	Chp-ox (μg/L)
t0	(1 h after starting)	<LOQ	C	28.52	<LOQ
			D	51.76	<LOQ
t24	Before maintenance	18.74 ± 7.52	C	<LOQ	0.096
			D	<LOQ	0.10
	After maintenance	No sampling	C	26.01	<LOQ
			D	20.68	<LOQ
t48	Before maintenance	62.86 ± 11.72	C	<LOQ	0.18
			D	<LOQ	0.23
	After maintenance	No sampling	C	21.56	<LOQ
			D	21.72	<LOQ
t72	Before maintenance	169.05 ± 41.47	C	<LOQ	0.17
			D	<LOQ	0.19
	After maintenance	No sampling	C	13.63	<LOQ
			D	27.61	<LOQ
t96		285.25 ± 34.61	C	< LOQ	0.21
			D	< LOQ	0.13

Fig. 1. Fulton's K indices at different time points (24, 48, 72, 96 h of CPF exposure and 7, 14, 21, 28 days after CPF exposure) in the negative unexposed group (blank), solvent-only exposed group and CPF-exposed group (50 μg/L). *Significant differences ($P < 0.05$) between unexposed/solvent-only and 96 hour-CPF exposure group (five specimens for each group).

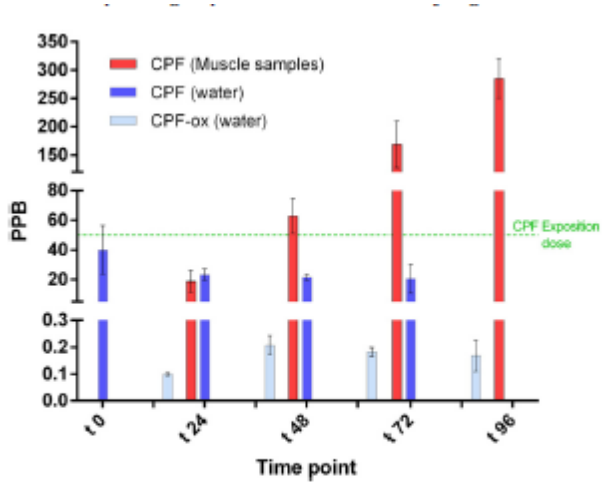

Fig. 2. Analytical determinations and concentrations of CPF and CPF-ox in muscle and water samples collected from the CPF-exposed group (50 μg/L). Analyte concentrations were reported as PPB (μg/L for water samples, ng/g for muscle samples).

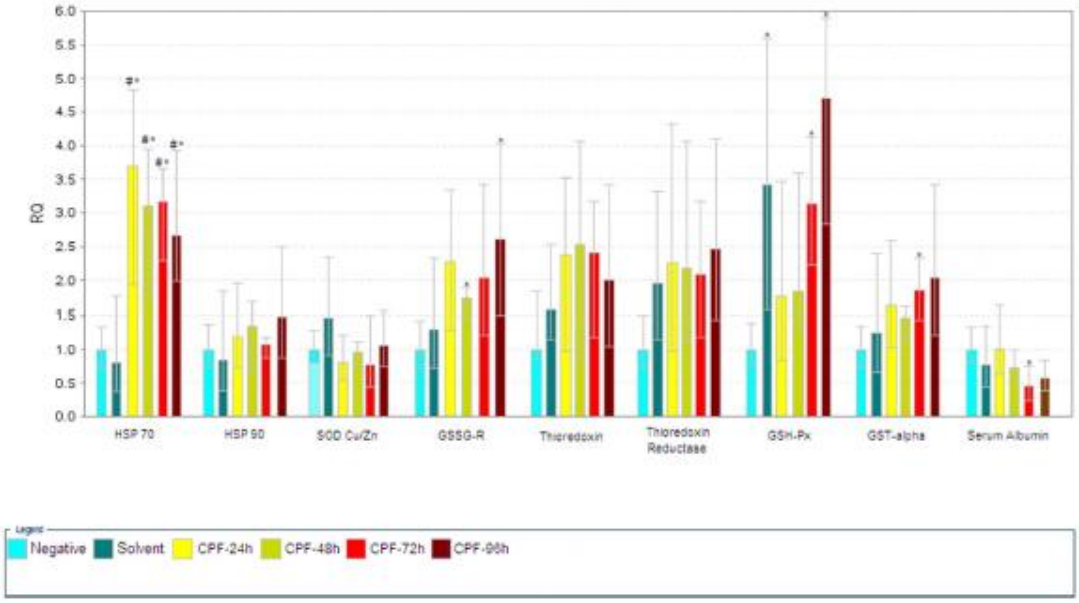

Fig. 3. Gene expression profiles in unexposed (Negative) and exposed (Solvent and CPF) groups in the first 96 h (five replicates for each group). Selected biomarkers: heat shock protein 70 and 90 (HSP70, HSP90), Cu/Zn isoform super oxide dismutase (SOD), glutathione reductase (GSSG-R), thioredoxin, thioredoxin reductase, glutathione peroxidase (GSH-Px), catalase (CAT), alpha isoform glutathione S-transferase (GST-alpha), serum albumin. *Significant differences ($P < 0.05$) between negative control group and exposed groups. #Significant differences ($P < 0.05$) between solvent-exposed group and CPF-exposed group.

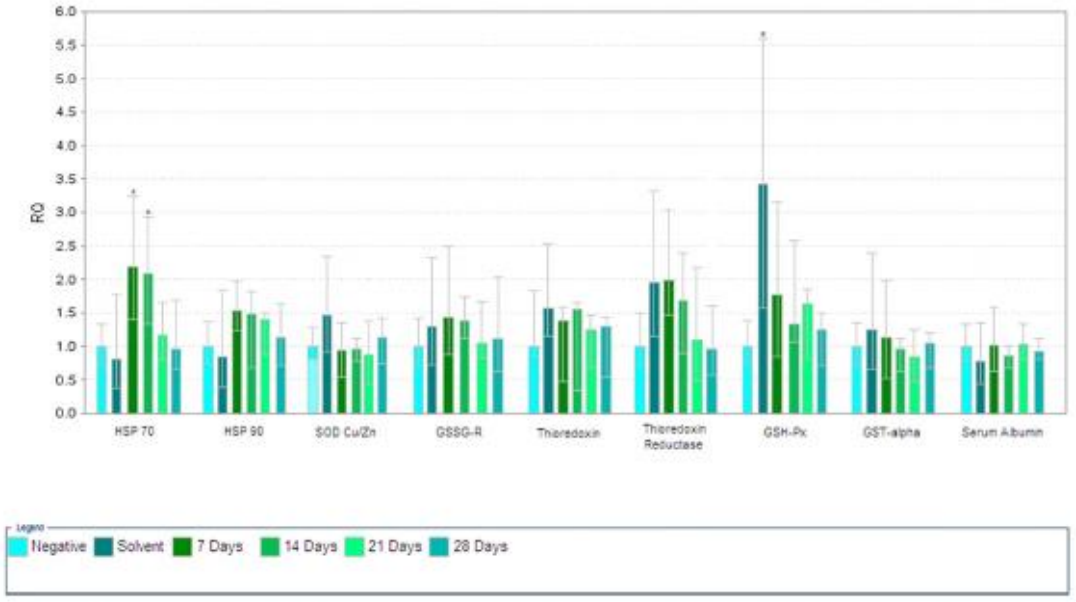

Fig. 4. Gene expression profiles in unexposed (Negative) and exposed (Solvent and CPF) groups in the subsequent 28 days after CPF exposure (five replicates for each group). *Significant differences ($P < 0.05$) between negative control group and exposed groups.