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Comparative evaluation of toxicological effects and recovery patterns in zebrafish (*Danio rerio*) after exposure to phosalone-based and cypermethrin-based pesticides

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ABSTRACT

This study evaluated the toxic effects and recovery patterns in zebrafish (*Danio rerio*) after exposure to phosalone-based (PBP) and cypermethrin-based (CBP) pesticides. Initially, the 96 h LC_{50} values of the pesticides were calculated as being 5.35 μg of active ingredient (AI) L^{-1} for CBP and 217 μg AI L^{-1} for PBP based on measured concentrations. Accordingly, experimental groups were exposed to three sublethal concentrations of pesticides for 96 h, separately, and then zebrafish were transferred to pesticide-free conditions for 10 and 20 days recovery periods. Biochemical markers were assessed including carboxylesterase (CaE), acetylcholinesterase (AChE), glutathione *S*-transferase (GST), lactate dehydrogenase, glutathione peroxidase, catalase, alanine and aspartate aminotransferase (ALT, AST) activities after the exposure and recovery periods. Also, the pesticide concentrations in test water were quantified by high-performance liquid chromatography (HPLC) analysis. Our results showed that AChE and CaE activities were significantly inhibited and GST was induced by both pesticides after 96 h exposure. For PBP exposure, the decreases for GST induction and CaE inhibition showed a partial recovery in pesticide-free conditions. However, the decreases in AChE activity for CBP exposure and insufficient increases in same enzyme activity for PBP exposure after 20 days in pesticide-free conditions indicated that the projected recovery period was not enough to the recovery of AChE activities and for the improvement of fish health.

1. Introduction

Recovery markers may have significance as environmental indicators when assessing the ecotoxicological risks of the pesticides for non-target organisms. Even pesticides applied to local areas, they may wash and carried away by irrigation waters and rains and currents to rivers and dam lakes and may be highly toxic to fish populations and also for other organisms, including human (Yhasmine, 2013). Therefore, many pesticides have been banned after determination of their toxic properties for non-target organisms, prolonged presence of toxic pesticide residues and also devastating effects on natural ecosystems.

The pesticides being used today generally have a short half-life in environmental conditions and show their effects in short time after application. For this reason, to predict the effects of pesticides on target and non-target organisms, test organisms have been exposed to tested materials for 96 h or less in various toxicological studies (Babu et al., 2014; Brodeur et al., 2016). However, pesticides may affect a lot of metabolic pathways, and cause to hazardous effects, even if they do not cause to lethality. The adverse effects of pesticides may also be continued for a longer time even if exposed organism moved to pesticide-free environment. Therefore, not only short-term effects of pesticides in a shorter exposure time but also long-term effects of pesticides after receiving the affected organisms to the pesticide-free environment in recovery period is vital for evaluation of environmental risks (Velki et al., 2017).

Two pesticides which from different groups, one is an organophosphate (OP), named phosalone (phosphorodithioate) (6-chloro-3-(diethoxyphosphinothioylsulfanylmethyl) − 1,3-benzoxazol-2-one) and the other is synthetic pyrethroid, called cypermethrin ([Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl) − 2,2-dimethylcyclopropane-1-carboxylate) were selected for the testing their toxicity in adult zebrafish.

Organophosphates are the basis of many pesticides and the advantages of OP pesticides is their low environmental persistence (Wogram et al., 2001). But, many studies showed the toxic effects or the presence of residues of phosalone in applied products. Phosalone exposed target/non-target organisms are presented its toxicity after a long time of application and far away from application areas due to runoff

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and wind-borne pollution (Oliveira et al., 2015) Therefore, phosalone has banned the pesticide in the European Community with Regulation 1376/07 (PAN, 2009). The usage of phosalone in Turkey was also prohibited. However, many reports indicate to keep using on agricultural application from developing/non-developing countries around the world (Huang et al., 2016). The other tested pesticide, cypermethrin is a type II pyrethroid, which has low persistence and toxicity to non-target organisms, which one of the top widely-used-five pyrethroids (Corcellas et al., 2015). It has also been extensively used in different crop productions for many years. Due to common uses, cypermethrin has been found in surface waters.

The fish toxicity tests as an experimental model help to assess toxicity potential of pesticides for aquatic organisms which is allowing early determination of effects of pollutants (Bonansea et al., 2016). Furthermore, zebrafish is a very suitable model organism for toxicity studies due to their easy maintenance and husbandry in laboratory conditions.

In order to assess the effects of xenobiotics on organisms, an important approach is to evaluate abnormal biochemical responses due to impaired physiological processes in the exposed organisms. These responses, which also called as biomarkers, have been used for evaluating of the sublethal effects of pesticides and, are important tools for the understanding of adaptation and recovery processes in environmental risk assessments (Ramesh et al., 2015). Acetylcholinesterase (AChE) and carboxylesterase (CaE) as a sign of pesticide intoxication; glutathione *S*-transferase (GST) as a detoxification enzyme; glutathione peroxidase (GPx) and catalase (CAT) as an antioxidant enzymes are the commonly used markers. Also, lactate dehydrogenase (LDH), aspartate and alanine aminotransferases (AST, ALT) may be suitable as metabolic biomarkers for evaluation health status of animals due to toxicity potential of pesticides (Jin et al., 2011; Rodriguez-Fuentes et al., 2015).

There are a lot of studies related to pesticide toxicity and biochemical changes after short-term exposure to pesticides in literature. However, the knowledge about changes in biochemical responses of commercial formulations of pesticides exposed non-target organisms in an aquatic ecosystem after their translocation to pesticide-free habitats was ignored. Therefore, the aims of the present study were: (1) to characterize the toxic effects of two pesticides on adult zebrafish, (2) to evaluate recovery profile of test organisms after exposed to single dose of the selected pesticides under controlled laboratory conditions, and finally, (3) to assessment of usefulness of selected biomarkers after exposure and recovery periods for environmental risk assessment studies.

2. Materials and methods

2.1. Chemicals

The commercial preparations of cypermethrin (25EC), and phosalone (35EC) for toxicity assays were purchased from a local agrochemical store. Active ingredients of the pesticides were labeled as 250 g L^{-1} for cypermethrin and 350 g L^{-1} for phosalone. Phosalone (36194, pestanal), cypermethrin (36128, pestanal), acetylthiocholine iodide (ACTI), p-nitrophenyl acetate (PNPA), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), DTT (1,4-Dithiothreitol), Bradford reagent, and bovine serum albumin (BSA) were purchased from Sigma Aldrich Chemical Company (MO, United States). β-nicotinamide adenosine-diphosphate reduced (NADPH) were provided from MP Biomedicals (United States).

2.2. Animals

About eight weeks old, juvenile zebrafish (*Danio rerio*) were purchased from a provider who imported the fish from commercial breeders in Singapore. Fish were acclimated for one month in Zebrafish Laboratory at Inonu University, in 120-L capacity tanks and only males were selected for the tests. One hundred zebrafish were maintained in each tank during acclimation periods. The water used in aquariums were prepared with 50% reverse osmosis water and 50% tap water and finally 3 g L^{-1} commercial sea salt were added to water. Aquariums were aerated with constant aeration and fish were maintained at 28 ± 1 °C and 14:10 h light/dark photoperiod. Fish were fed twice daily with commercial flake food (Tropical D-50 Plus, Germany) ad libitum. Water was tested for total chlorine, conductivity and total nitrite and nitrate levels, and 10% of water was changed every two days periodically. Organisms were used for toxicity tests after the acclimation period. The research protocol (Protocol No. 2012/A-73) for animals in the experiments was reviewed and approved by the Inonu University Research Animals Ethics Committee.

2.3. Toxicity tests

Static renewal toxicity tests were performed in the 5 L capacity polycarbonate tanks containing 4 L test solution. Test media of controls and all exposure groups were prepared using test water prepared as described above. All pesticide solutions were prepared daily in test water. The fish were exposed to pesticide solutions at 28 °C (± 1 °C) with a 14:10-h light: dark photoperiod in static test conditions. Aeration was provided for each tank during the experiments.

2.3.1. Determination of LC_{50} values

Fish were exposed to seven concentrations of cypermethrin-based pesticide (actual CBP concentrations were 2.82–10.5 μg AI L^{-1}) and six concentrations of phosalone-based pesticide (actual PBP concentrations were 86–505 μg AI L^{-1}). All concentrations were tested with two replicates with seven fish in each tank, using a total of 14 fish which were selected randomly. Test solutions were changed every 24 h during the 96 h test periods to keep known concentrations of pesticides. Fish were not fed during the exposure period. The dead animals were removed, and the numbers were recorded. At the end of the experiment, median lethal concentrations (LC_{50}) were determined for 96 h (also if possible for 48 and 72 h) exposure periods.

2.3.2. Exposure and recovery tests

Fish were exposed to three sublethal measured concentrations of PBP (2.87, 3.45 and 4.41 μg AI L^{-1}) and CBP (12.5, 44.8 and 98.6 μg AI L^{-1}). At the beginning of the exposure, conductivity and oxygen levels for control and exposure groups were measured as 0.53 mS cm^{-1} (range of 0.42–0.61), and 8.8 mg L^{-1} (range of 6.5–11.4), respectively. The pH was measured as a tolerable range (6.5–8.5) during the tests. All groups were tested with four replicates in test tanks, using a total of 56 fish. Fish were not fed during the exposure period. After 96 h exposure period, four fish from each test tank were collected (totally 16 fish for each test group) for biochemical studies. Remaining fish were transferred into clean water for recovery studies. Collected fish samples were placed into microcentrifuge vials; fish were chilled on ice and stored in a − 80 °C freezer until the enzymatic assays. Remaining fish from each exposure group were kept in fresh, pesticide-free water for 10 or 20 days for recovery periods. Fish were fed twice daily during recovery periods. At the end of each period, the animals were collected and stored in ultra-freezer after collection methods described previously.

2.4. Biochemical studies

2.4.1. Preparation of enzymatic sample

The fish samples were thawed on ice, weighed and homogenized (1:5, w/v) in ice-cold 0.1 M K-phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA and 1 mM DTT with using a polytron homogenizer. The homogenates were centrifuged at 16,000 × *g* for 20 min at 4 °C, and supernatants were transferred into clean microfuge tubes. After the centrifugation procedures, enzyme activities in the post-

mitochondrial fraction were assayed as soon as possible, on the same day without freezing of the supernatant.

2.4.2. Determination of enzyme activities

The all enzyme activities were assayed spectrophotometrically with some modifications for microplate system (VersaMax, Molecular Devices Corp., USA) at 25 °C in triplicate and expressed as specific activity except CAT. Only the activity of CAT was determined using spectrophotometer in quartz cuvettes (Shimadzu, UV-1601).

The activities of AChE was assayed using ACTI as a substrate, as described by Ellman et al. (1961). Ten microliters of supernatant were used for each well and the final concentrations of ACTI and DTNB, which prepared in Trizma buffer (pH 8.0; 0.1 M), were 0.7 mM and 0.14 mM, respectively. Enzyme kinetics were monitored at 412 nm for 1 min.

The CaE activity was determined according to the procedure of Santhoshkumar and Shivanandappa (1999). 5 µl of supernatant and 250 µl 0.05 mM Trizma (pH 7.4) was incubated for 3 min. The reaction was started by the addition of 5 µl PNPA (26 mM) as the substrate. The liberated *p*-nitrophenol was followed at 405 nm for 2 min.

The GST activity was assayed after formation of GSH conjugate with CDNB (Habig et al., 1974). The mixture contained 10 µl supernatant, 0.1 M K-phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CDNB. Change in absorbance was monitored at 344 nm for 2 min.

The GPx activity was detected according to Stephensen et al. (2002) by microplate assay with modifications. Twenty microliters of supernatant and 160 µl of the assay mixture were added to each well. The assay mixture contained 0.1 mM NADPH, 1 mM GSH, 2 mM sodium azide, 1 mM EDTA, 50 mM phosphate buffer (pH 7.4) and 0.5 U glutathione reductase. This mixture was kept for 6 min at 25 °C, and the reaction was started by addition of H_2O_2 (20 µl, 2.5 mM). The NADPH oxidation rate was measured during 3 min at 340 nm.

CAT activity was assayed using method described by Aebi (1974) at 240 nm for determination of decrease in absorbance. The reaction solution contained 20 µl of supernatant, 480 µl 12.5 mM H_2O_2 and K-phosphate buffer (0.05 M, pH 7.0), in a total volume of 1 mL.

The LDH, AST, and ALT activities were determined using the commercial assay kits (Biolabo, France). 5 µl or 10 µl of supernatant for LDH or AST/ALT assays were transferred into each well. The reaction started adding 200 µl of reaction solution into wells using a multi-channel pipette and changes in absorbance was monitored at 340 nm according to methods described in the manufacturers' test manuals.

The concentrations of total protein in each supernatant were assayed using the Bradford reagent (Bradford, 1976) and bovine serum albumin (BSA) was used as a standard protein source. 5 µl of diluted (1:4) supernatant as protein source and 250 µl Bradford reagent was dispensed. Absorbance was read at $\lambda = 595$ nm, and the protein concentration was calculated from the calibration curve constructed from providing of the serial dilutions of BSA standard (0–1.4 mg BSA mL^{-1}). The total protein amounts for each sample were used to determine the specific activity (nmol min^{-1} mg protein $^{-1}$) of each enzyme.

2.5. HPLC analysis of pesticides

The actual concentrations of tested pesticides in the exposure media determined using a HPLC system (Thermo Finnigan Surveyor, USA) containing a diode array detector (DAD) with Zorbax Eclipse XDB-C_{18} column (5 µm × 4.6 mm internal diameter, and 250 mm length). For both pesticides, the column was eluted with a mixture of mobile phase and was quantified by comparing with standard curves of known pesticide concentrations. Phosalone and cypermethrin standards (pesnatal) were used to quantify by standard calibration using HPLC-DAD. The solvents were 80% methanol: 20% phosphate buffer (pH 5; 25 mM) (v/v) or 80% acetonitryl: 20% ultra-pure water (v/v) for phosalone or cypermethrin standards, respectively. The phosalone and cypermethrin determined at 234 or 211 nm, respectively. The flow rate was 1 mL min^{-1} for both pesticides. The detection limits (LOD) and the limit of quantification (LOQ) were 9 µg $L^{-1}$and 29 µg $L^{-1}$for phosalone, and 9 µg $L^{-1}$and 32 µg L^{-1} for cypermethrin. The actual pesticide concentrations in exposure solutions were calculated for freshly prepared exposure media and for 24 h-aged exposure media using HPLC analysis. Furthermore, higher concentrations of commercial formulations measured in both exposure solutions and pesticide-specific-solvent to assess whether the lower concentrations which measured in the previous experiment were due to the measurement limits or the solubility (data were presented in supplementary material at Fig S1).

2.6. Data analysis

Assessment of lethal toxicity was made by fitting a sigmoidal logistic curve using the trial version of SigmaPlot software (ver. 14.0; Systat Software Inc., USA) from which median lethal concentrations (LC_{50}) were calculated. Statistical analyses of biochemical markers were performed using GraphPad Prism (Ver. 5.0; Graph Pad Software Inc, USA). Data tested initially for determination of homogeneity of variances and normality by the Bartlett's and Shapiro-Wilk tests, respectively. Nonparametric data were analyzed using Kruskal–Wallis followed by Dunn's test. Parametric data were analyzed using the One-way Analysis of Variance (ANOVA), followed by the Tukey's test. Differences were considered significant at $p < 0.05$.

3. Results

The mortality data obtained from the toxicity tests are presented in Fig. 1. The LC_{50} values calculated based on measured concentrations of active ingredient (AI) of pesticides. The 96 h LC_{50} values were calculated as 217 µg AI L^{-1} for PBP and 5.35 µg AI L^{-1} for CBP. Also, the 48 h and 72 h LC_{50}s for CBP were determined as 7.23 µg AI L^{-1} and 5.44 µg AI L^{-1}, respectively. However, we could not be able to calculate 48 h and 72 h LC_{50}s for PBP.

Phosalone and cypermethrin presence in the exposure media were monitored during the experiments and confirmed by HPLC with the same retention time as the commercial standard (Fig. S1). According to the results of HPLC measurements, both phosalone and cypermethrin levels in the exposure solutions did not change significantly within 24 h (Table 1). The HPLC-determined concentrations in exposure solutions were significantly low (approx. 30% and 7%) for cypermethrin and phosalone comparing with our calculated nominal concentrations of the commercial products. The nominal concentrations selected as 50 µg AI L^{-1}, 100 µg AI L^{-1} and 500 µg AI L^{-1} were measured as 16 µg AI L^{-1}, 29 µg AI L^{-1} and 136 µg AI L^{-1} in the exposure water, respectively for CBP. Also, for PBP, nominal concentrations selected as 500 µg AI L^{-1}, 1000 µg AI L^{-1}, 2254 µg AI L^{-1} and 5000 µg AI L^{-1} were measured as 39 µg AI L^{-1}, 117 µg AI L^{-1}, 170 µg AI L^{-1} and 474 µg AI L^{-1}, respectively in exposure water. The measured pesticide concentrations in organic solvents were found to be higher than the concentrations in exposure media (approx. 80% and 22% of the nominal concentrations) for cypermethrin and phosalone because their water solubility is very low (Fig. S1).

The activities of selected biomarker enzymes after 96 h exposures to PBP or CBP and also the enzyme activities of fish after 10 and 20 days recovery periods presented in Table 2 and Table 3, respectively. All concentrations of both pesticides caused to inhibition of esterases (both AChE and CaE). The AChE inhibition levels were determined between 89% and 95% for 96 h PBP exposure. The AChE activity was still inhibited 76–97% after 10 days of the recovery period, and it found 76–98% inhibited after 20 days of recovery periods on surviving fish compared to control animals. The assayed ratio of AChE activities were less than 25% of control fish due to 96 h PBP exposure and also during recovery periods ($p < 0.001$). Distinctively, AChE inhibition ratio increased in recovery periods in CBP exposure groups comparing with PBP exposure groups. AChE inhibition levels determined between 14%

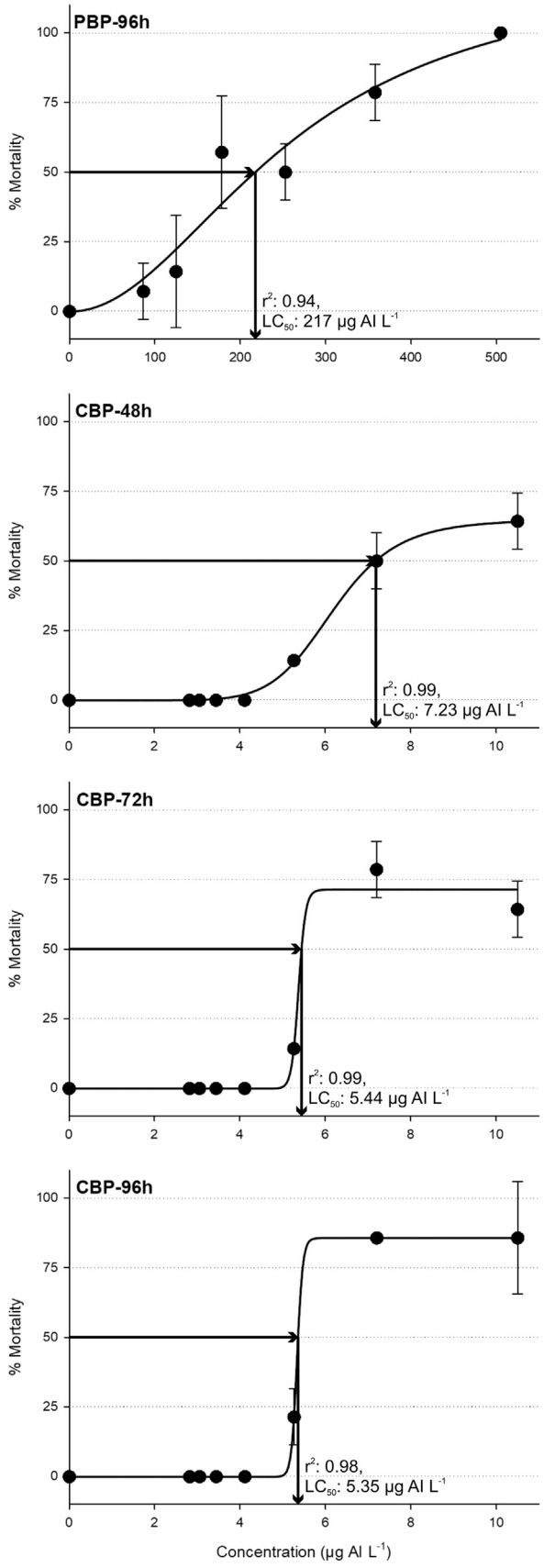

Fig. 1. Mortality of zebrafish after exposure to phosalone-based pesticide (PBP) and cypermethrin-based pesticide (CBP). Curve represents the non-linear fitting of mortality data (sigmoidal). Circles represent mean experimental mortality data ± standard errors.

Table 1
Measured phosalone and cypermethrin concentrations in exposure water after HPLC analysis.

Pesticide	Nominal conc. (µg AI L^{-1})	Measured conc. (µg L^{-1})					
		0 h			24 h		
Phosalone	10	nd			nd		
	50	nd			nd		
	100	nd			nd		
	500	39	±	1	32	±	3
	1000	117	±	9	–		
	2254	170	±	8	167	±	11
	5000	474	±	1	–		
Cypermethrin	10	nd			nd		
	14.4	nd			nd		
	50	16	±	2	nd		
	100	29	±	4	26	±	5
	500	136	±	34	142	±	5

The measured concentrations were expressed as mean ± standard deviation. nd: pesticide was not detected or below instrument detection limit (detection limits for phosalone: LOD = 9 µg L^{-1}, LOQ = 29 µg L^{-1}; for cypermethrin: LOD = 9 µg L^{-1}, LOQ = 32 µg L^{-1}).

and 81% for 96 h CBP exposed animals, but the ratio of inhibition of the enzyme determined between 48% and 83% after 10 days recovery and 62–90% after 20 days recovery periods.

The level of inhibition of CaE was higher for PBP groups according to CBP exposed fish. However, differently, CaE inhibition levels in PBP exposure significantly decreased in recovery periods on exposed to 2.87 and 3.45 µg AI L^{-1} concentrations of the pesticide. CaE inhibition ratios determined between 78% and 84% for 96 h PBP exposure, between 41% and 86% after 10 days recovery and between 19% and 86% after 20 days of recovery. On the other hand, CaE inhibition levels were calculated for CBP between 47% and 71% for exposure, between 47% and 65% after 10 days and between 37% and 78% after 20 days of recovery periods (Table 3).

As a detoxification enzyme, GST activity significantly increased after 96 h PBP exposure and 10 days of recovery period in almost all tested concentrations ($p < 0.001$). However, the increase ratio of enzyme activity was between 60% and 96% for 96 h PBP exposure. The GST activity was higher 12–57% as for that controls after 10 days of recovery and 6–24% after 20 days of recovery periods. GST induction rate finds similar for 12.5 and 44.8 µg AI L^{-1} concentrations of CBP for 96 h exposures (84% and 80%, respectively). However, highest CBP concentration caused to significant GST inhibition, and inhibition ratio decreased with 20 days recovery of fish.

The activities of two oxidative stress enzymes (GPx and CAT) significantly induced after 96 h exposure due to 3.45 and 4.41 µg AI L^{-1} exposure concentrations of PBP ($p < 0.05$). The induction of CAT continued for 3.45 and 4.41 µg AI L^{-1} concentrations on PBP exposed fish ($p < 0.05$ and $p < 0.001$, respectively) on 10 days of recovery. The CAT activity did not change significantly before and after recovery periods in animals exposed to a concentration of 2.87 µg AI L-1 of PBP. However, in animals exposed to the concentration of 4.41 µg AI L^{-1} PBP, CAT activity was not changed on ten-days-recovery but significantly reduced after 20 days of recovery (Table 3).

The LDH and AST activities significantly inhibited after PBP exposure in 96 h. The activities of the enzymes also remained in suppressed after recovery periods, in general. The 96 h PBP exposure induced ALT activity for all exposed concentrations, but the rate of induction relatively decreased with recovery periods. The increase levels of ALT activity determined between 156% and 183% after 96 h, between 121% and 142% on 10 days recovery and between 109% and 121% on 20 days recovery due to PBP exposure. Similar to PBP exposure, AST activity significantly decreased but, ALT activity increased after 96 h of CBP exposure. However, the changes in enzyme activities were not dose related.

Table 2
The enzyme activities of zebrafish after phosalone-based pesticide (PBP) exposure and recovery periods.

			BIOMARKER ENZYMES																														
			Esterases									Detoxification and Oxidative Stress Enzymes												Other Metabolic Enzymes									
		Measured Conc. (μg AI L^{-1})	AChE				CaE					GST				GPx				CAT				LDH				AST				ALT			
Exposure period	96 h	Control	437	±	24.3	a	659	±	24.8	a		34.7	±	1.4	a	20.1	±	2.8	a	26.5	±	2.25	a,b	607	±	42.5	a	414	±	37.3	a	27.0	±	1.25	a
		2.87	49.7	±	3.8	b	139	±	11.0	b,c		63.9	±	4.3	b,c	12.1	±	2.0	a	22.0	±	1.70	a	421	±	15.5	b	312	±	19.5	a,b	42.2	±	1.83	b
		3.45	30.0	±	2.0	b,c	146	±	6.3	b		68.1	±	3.0	b	29.7	±	2.9	b,c	35.2	±	2.96	b,c	445	±	34.5	b	249	±	26.0	b	47.4	±	2.10	b
		4.41	19.8	±	2.3	c	109	±	4.3	c		55.5	±	1.6	c	35.5	±	3.1	c	41.2	±	2.94	c	758	±	37.3	a	273	±	14.5	b	49.3	±	4.48	b
Recovery period	10 days	Control	631	±	38.3	a	784	±	55.9	a		52.1	±	2.4	a	21.7	±	5.5	a	20.5	±	1.88	a	672	±	33.9	a	331	±	22.0	a	38.3	±	2.03	a
		2.87	149	±	15.6	b,c	466	±	49.6	a,b		70.2	±	2.6	b,c	9.2	±	1.2	b	29.1	±	2.56	a,b	507	±	25.5	a	292	±	17.4	a	46.8	±	2.95	a,b
		3.45	127	±	17.4	c	421	±	55.3	b		81.7	±	4.5	b	23.8	±	3.0	a	40.0	±	4.27	b	536	±	34.4	a	293	±	21.4	a	54.3	±	3.82	b
		4.41	18.0	±	2.5	d	109	±	7.6	c		58.5	±	2.6	a,c	24.1	±	3.8	a	21.4	±	2.23	a	339	±	13.7	b	189	±	13.7	b	46.5	±	1.78	a,b
	20 days	Control	606	±	39.1	a	756	±	39.2	a		57.8	±	3.2	a	14.1	±	2.1	a	25.9	±	2.74	a,b	877	±	55.9	a	371	±	19.9	a	41.2	±	1.77	a,c
		2.87	147	±	13.5	b	615	±	59.8	a,c		71.8	±	1.8	b,c	29.5	±	2.4	b	33.0	±	1.72	a	513	±	17.9	b	275	±	24.2	b	45.0	±	2.05	a,b
		3.45	118	±	20.1	b	322	±	43.5	b,c		67.4	±	2.3	a,c	23.3	±	2.4	a,b	31.1	±	2.59	a	428	±	29.5	b,c	270	±	21.7	b	34.7	±	1.69	c
		4.41	13.3	±	1.7	c	109	±	10.4	b		61.1	±	0.7	a	29.4	±	3.6	b	19.1	±	1.25	b	401	±	12.5	c	230	±	12.3	b	50.0	±	1.35	b

All enzyme activities were expressed as nmol min^{-1} mg $protein^{-1}$ ± standard error. AChE: acetylcholinesterase, CaE: carboxylesterase, GST: glutathione *S*-transferase, GPx: glutathione peroxidase, CAT: catalase, LDH: lactate dehydrogenase, AST: aspartate aminotransferase, ALT: alanine aminotransferase.
Each concentration was tested with 4 replicates with 16 fish in total. Different letters denote significant differences within treatments ($p < 0.05$).

Table 3
The enzyme activities in zebrafish after cypermethrin-based pesticide (CBP) exposure and recovery periods.

			BIOMARKER ENZYMES																														
			Esterases									Detoxification and Oxidative Stress Enzymes												Other Metabolic Enzymes									
		Measured Conc. (μg AI L^{-1})	AChE				CaE					GST				GPx				CAT				LDH				AST				ALT			
Exposure period	96 h	Control	437	±	24.3	a	659	±	24.8	a		34.7	±	1.4	a	20.1	±	1.8	a	26.5	±	2.25	a	607	±	42.5	a	414	±	37.3	a	27.0	±	1.25	a
		12.5	375	±	74.1	a,c	350	±	87.1	b		62.6	±	2.7	b	19.3	±	2.2	a,b	30.3	±	2.39	a	672	±	32.5	a	328	±	18.9	a,b	35.9	±	1.74	b
		44.8	124	±	13.2	b,c	156	±	12.1	b		63.7	±	2.2	b	11.4	±	1.7	b	26.5	±	1.63	a	640	±	32.3	a	273	±	12.3	b	35.7	±	1.32	b
		98.6	81.6	±	10.4	b	193	±	13.4	b		14.8	±	0.9	a	17.0	±	5.2	a,b	25.1	±	1.68	a	649	±	28.0	a	327	±	19.5	a,b	32.8	±	2.10	a,b
Recovery period	10 days	Control	631	±	38.3	a	785	±	55.9	a		52.1	±	2.4	a	21.7	±	5.5	a,b	20.5	±	1.88	a	672	±	33.9	a	331	±	22.0	a,b	38.3	±	2.03	a,b
		12.5	325	±	59.7	b	355	±	67.2	b		55.8	±	1.5	a	27.6	±	3.0	a	19.4	±	1.13	a	777	±	46.5	a	356	±	24.2	a	39.9	±	2.61	a,b
		44.8	157	±	26.0	b	419	±	58.5	b		72.9	±	2.2	b	15.4	±	2.3	b	21.1	±	1.23	a	739	±	43.2	a	194	±	41.3	b	38.9	±	2.26	b
		98.6	106	±	16.0	b	274	±	30.4	b		16.8	±	1.2	c	13.4	±	2.7	b	24.4	±	1.98	a	785	±	42.1	a	346	±	21.4	a	51.4	±	3.73	a
	20 days	Control	606	±	39.1	a	756	±	39.2	a		57.8	±	3.2	a	14.1	±	2.1	a	25.9	±	2.74	a	877	±	55.9	a	371	±	19.9	a	41.2	±	1.77	a
		12.5	230	±	43.8	b	474	±	68.8	a,c		51.7	±	1.8	a	19.9	±	2.5	a	28.3	±	1.34	a	639	±	42.1	b,c	222	±	23.3	b	33.8	±	2.19	b
		44.8	183	±	38.3	b	336	±	55.2	b,c		80.1	±	2.9	b	44.1	±	3.1	b	24.7	±	1.81	a	819	±	28.7	a,c	345	±	16.4	a	48.4	±	1.81	a
		98.6	60.9	±	14.4	b	169	±	20.0	b		54.4	±	4.3	a	10.0	±	2.3	a	14.9	±	1.51	b	505	±	25.2	b	226	±	18.5	b	23.8	±	2.54	c

All enzyme activities were expressed as nmol min^{-1} mg $protein^{-1}$ ± standard error. AChE: acetylcholinesterase, CaE: carboxylesterase, GST: glutathione *S*-transferase, GPx: glutathione peroxidase, CAT: catalase, LDH: lactate dehydrogenase, AST: aspartate aminotransferase, ALT: alanine aminotransferase.
Each concentration was tested with 4 replicates with 16 fish in total. Different letters denote significant differences within treatments ($p < 0.05$).

4. Discussion

Ecotoxicological risk assessment is important part of pesticides application but determination of recovery of pesticide toxicity is also important for forecasting of environmental health on non-target organisms. The aquatic environment is polluted by a variety of anthropogenic compounds that pesticides are one of the most common and highly toxic substances for fish and other organisms especially in aquatic ecosystems. The existing pesticides show their main toxic effects in the short term after application and/or exposure due to their specific action modes in their relatively short half-life. Furthermore, sublethal concentrations of available pesticides that do not cause to death over the short term but do harm the individual, thus making it expend resources to survive in a state of altered equilibrium (Di Giulio and Hinton, 2008). Due to this approach, knowledge of short-term toxicity after exposure and sub-lethal effects in the recovery of toxicity are important to evaluate for environmental risks of pesticides on non-target organisms. Many pesticides have low water solubility which results in bio-concentration into organisms from surrounding matrices such as water (Muir et al., 1985) and toxicity may increase due to their bio-concentration on organisms. In the present study, as a model organism, exposure of adult zebrafish to the sub-lethal concentration of commercial formulations of phosalone or cypermethrin showed significant alterations in selected biochemical markers. On the other hand, symptoms of toxic effects did not significantly change on adult zebrafish due to the 10 and 20 days of the recovery periods for both pesticides.

Commercial formulations of these pesticides, which also cause environmental problems as a result of agricultural activities, have been tested in this study. These pesticides, which contain active ingredients as well as many other preservatives and solvents, are usually used after mixing in water, without the use of organic solvents. Insufficient dissolution of the pesticides in water may cause nominal and measured concentrations to be different from each other. Because of this reason, we tried to determine the effective concentrations of the tested pesticides in this study.

Phosalone was classified as moderately hazardous pesticide (class II) according to WHO (2010) and it is banned in EU countries (PAN, 2009) but still one of the common used pesticide on fruit crops and other agricultural products around the world. Our results show that the 96 h LC_{50} of PBP determined as 217 μg AI L^{-1}. The literature also represents the range of PBP for 96 h LC_{50} values for other fish species (*Oreochromis mossambicus*, *Heteropneustes fossilis*, *Lepomis marcrochirus*, *Oncorhynchus mykiss,* etc.) between 83 μg L^{-1} to 3400 μg L^{-1} (Ali and Rani, 2009; TOXNET, 2008). However, phosalone is weakly dissolved in water and the calculated amount by HPLC is significantly less than our calculated nominal concentration in exposure media. Indeed, the calculated phosalone concentration was less than %10 of the nominal concentration in exposure solutions because commercial formulations have also different compounds such as surfactants, solvents and preservatives in the commercial formula (Damiens et al., 2004). Even though the case, poorly soluble material has still high lethal effects on adult zebrafish, in this study.

Organophosphates (OP) show their toxic effect with AChE inhibition (Sancho et al., 2000). Although B-esterases being used to evaluate exposure to OP pesticides and inhibition patterns of these enzymes have studied in aquatic organisms, their recovery patterns are unknown for most species (Barata et al., 2004). The duration of cholinesterase depression after exposure to AChE inhibitor affects the recovery of the organisms and possibility to survive in their environment (Ferrari et al., 2004). The AChE activity inhibited 76–98% on exposed fish compared to control animals while they are still alive and, the inhibition levels also increased dose-related exposure, as it was expected, in this study. On the other hand, total AChE inhibition on fish exposed to different concentrations of phosalone had a risk of survivability still continued after even 20 days of recovery periods. Furthermore, unsurprisingly, 20 days pesticide-free condition for fish did not provide significant recovery for AChE activity. The CaEs are also irreversibly inhibited by organophosphates presumably by formation of an irreversible covalent bond between the catalytic serine of enzyme and OPs (Hatfield et al., 2016). The CaE inhibition showed a clear dose–response relationship with increased PBP concentrations. It is known that the most of the OP pesticides are metabolized to dialkyl phosphates and CaEs have a protective mechanism by irreversibly binding the active metabolites of the OPs and therefore preventing it from reaching its primary target, AChE (Jokanovic, 2001). However, the recovery levels of CaE activity were higher than AChE activity on 2.87 and 3.45 μg AI L^{-1} PBP exposures duration of the recovery. Further CaE recovery levels as far as than AChE recovery may relate to the higher *de novo* synthesis of the enzyme activity. The CaE has also multiple isozymes that some of them are not sensitive to OP inhibition (Wheelock et al., 2005).

The GST activity increased 160–196% compared to control fish. On the other hand, enzyme activity was still higher comparing control animals after 10 days and 20 days of recovery. About 50% decrease of enzyme induction with recovery periods may represent that recovery is positively affected fish health but not enough to restore normal healthy conditions on sub-acutely exposed animals (Ramesh et al., 2015). The results also suggest that esterases and GST may be useful biomarkers for monitoring of PBP exposure and duration of recovery of exposed animals.

The induction of GPx and CAT may provide a first line defense against radical oxygen species (ROS) (Kavitha and Rao, 2007). These enzyme activities showed a dose- related induction after exposed to 3.45 and 4.41 μg AI L^{-1} concentrations of PBP within 96 h. Although, CAT induction relatively decreased in lower exposure concentrations (2.87 and 3.45 μg AI L^{-1}) after 20 days of recovery time, and highest exposure concentration (4.41 μg AI L^{-1}) also caused to inhibition of enzyme activity. Oxidative stress is believed to occur when there is an imbalance in the biological oxidant-to-antioxidant ratio, and CAT activity increased during exposure periods as a response to toxicant stress and serves to neutralize the impact of increased ROS generation (Patil and David, 2013). Transferring of the PBP exposed fish to pesticide-free life condition exhibited a recovery for CAT activity. However, the inhibition of enzyme activity observed at the highest exposure concentration (4.41 μg AI L^{-1}) even after 20 days of recovery may be related to the superoxide radicals in the environment (Slaninova et al., 2009).

The LDH and AST activities were significantly decreased after 96 h PBP exposure and for during both recovery periods. However, the 96 h exposure of PBP caused to induction of ALT activity in all exposure groups but, induction rate of the enzyme activity relatively decreased the duration of the recovery. Similar results reported for AST and LDH inhibition, but ALT activity showed increment in *Heteropneustes fossilis* after exposure to chlorpyrifos (Shoaib and Siddiqui, 2016; Tripathi and Shasmal, 2010). The LDH inhibition may indicate the reduction of anaerobic capacity in response to pesticide treatment (Tripathi and Shasmal, 2010). The highest LDH inhibition rate for PBP exposure groups determined after 20 days recovery period. Thus PBP-induced impairment in anaerobic (LDH) and amino acid metabolism (AST, ALT) cannot be repaired in recovery periods on zebrafish adults.

Cypermethrin which is a pyrethroid that is explained as fish are poor ability to degrade and metabolize the pesticide and the calculated LC_{50} for 96 h exposure on adult zebrafish is 5.35 μg AI L^{-1}. The cypermethrin is one of the extremely toxic pesticides to fish and 96 h LC_{50} concentration of cypermethrin is reported between 2.66 and 400 μg L^{-1} for different fish species (*Odontesthes bonariensis*, *Channa punctatus*, *Anabas testudineus*, *Cnesterodon decemmaculatus,* etc.) (Babu et al., 2014; Brodeur et al., 2016; Carriquiriborde et al., 2009). Cypermethrin is also one of the known pollutants for surface water which concentration may increase up to 2.8 μg L^{-1} (Jaensson et al., 2007). For this reason, one of the concentrations likely to be present in the aquatic environment is tested 2.87 μg AI L^{-1}. The HPLC-calculated concentrations of the

cypermethrin in aqueous media were relatively high which 70% of the nominal concentration calculated in aqueous media with compared to PBP. On the other hand, calculated 96 h LC_{50} was very low for cypermethrin that it shows commercial CBP has still high toxicity on adult zebrafish.

The CBP inhibits esterases such as OP pesticides, but the main action is not related to cholinesterase inhibition (Vani et al., 2012). Similar to other pyrethroids, cypermethrin as a Type II pyrethroid leads to slow activation and inactivation of sodium ion channels. Rao and Rao (1995) speculated that brain AChE in cypermethrin exposed rat may inhibit a due interaction between this highly hydrophobic pyrethroid and the hydrophobic aromatic surface region of AChE. In our study, inhibition levels of AChE activity significantly increased after CBP exposure in 10 and 20 days of recovery periods. Furthermore, similar to AChE inhibition, as seen after PBP exposure in zebrafish, high inhibition ratios in AChE activity with CBP exposure may explain with irreversible inhibition and delayed *de novo* synthesis of enzyme protein. On the other hand, irreversible AChE inhibition may also relate to the formation of metabolites such as 3-phenoxybenzoic acid and 3-(2′,2′-dichlorovinyl) – 2,2-dimethylcyclopropane carboxylic acid which is also known as endocrine disruptors (Yao et al., 2015). The cypermethrin is stable to hydrolysis with a half-life of greater than 50 days in neutral acid/base water conditions and it may accumulate in fish tissues which may inhibit AChE activity even if the fish can move to pesticide-free conditions (Corcellas et al., 2015; Mantzos et al., 2016)

After 96 h exposure and duration of 10 days recovery, two lower exposure concentrations (12.5 and 44.8 μg AI L^{-1}) of CBP caused to induction in GST activity about 180% and 100–140% compared to controls, respectively but, higher exposure concentration (98.6 μg AI L^{-1}) inhibited the enzyme activity. Exposure to sublethal concentrations of CBP may cause enzyme induction probably due to detoxification of xenobiotic, and this still continued in 10 days recovery may be related to irreversible deterioration in pesticide metabolism. On the other hand, high concentrations of pesticide may cause of impairment of general metabolism of fish that the inhibition of GST activity may reflect the consequence of toxicity. Similar results were understood toxicity and/or recovery process in animals additional to also reported in frog tadpoles after exposure to relatively high concentrations of cypermethrin which caused 38% mortality in tadpoles (Greulich and Pflugmacher, 2004). Therefore, higher sublethal exposure concentrations to CBP may limit phase II detoxification process on fish due to cellular damage that it may be responsible for the dysfunction of the GST.

On the other hand, 96 h CBP exposure caused to ALT induction while AST activity significantly inhibited that this adverse effect in transaminases may relate to enhanced protein catabolism and hepatocellular damage in the organism (Begum, 2005). Kumar et al. (2011) asserted in their study that, the significant decrease in the levels of amino acids concomitant with remarkable increase in the activities of ALT and AST in fish species elucidated the amino acid catabolism as one of the main mechanism of meeting out the immediate energy demand of the fishes in condition of cypermethrin exposure.

5. Conclusion

Results of this study indicate that exposure to lethal and sublethal concentrations of two different common commercial pesticides caused alterations in the enzymatic parameters of zebrafish. The data about recovery suggest that the toxicity caused by tested pesticides is reversible to some extent with some enzymes show normalization after 20 days recovery periods. However, insufficient recovery or deterioration for some biomarkers indicates that pesticide exposure may cause to unfavorable changes in exposed fish and these changes may continue even long after the termination of exposure. Consequently, our study shows the alterations of individual biomarker enzyme may provide an early warning signal for evaluation pesticides toxicity, but also co-evaluation of biomarker responses may be sufficient for assessment toxic effects on aquatic organisms in environmental conditions

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.05.055.

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