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Response of Acetylcholinesterase (AChE) in the Erythrocyte and Liver of Rainbow Trout Exposed to Carbosulfan

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Abstract

Present study was conducted to determine the effects of long term carbosulfan exposure on erythrocyte and liver acetylcholinesterase (AChE) activity in rainbow trout (*Oncorhynchus mykiss*), and to assess sensitive tissue to carbosulfan in terms of AChE activity. For these purpose, fish were allowed to recover in toxicant-free water for 24 days after 60 days of exposure. AChE activity was determined spectrophotometrically using acetylthiocholine iodide as substrate in the erythrocyte and liver. Erythrocyte and liver AChE of carbosulfan-exposed fish showed considerable inhibition rate. A higher degree of enzyme inhibition was observed in the erythrocyte when compared with liver. The degree of enzyme inhibition had a positive correlation with the time of exposure. Erythrocyte and liver AChE activities were recovered after 18 d and 21 d, respectively. Results indicate that the erythrocyte AChE activity is more sensitive to carbosulfan exposure than that of liver AChE. The greater sensitivity of the erythrocyte AChE suggests that it may be more useful as a biomarker of carbosulfan exposure or contamination.

Keywords: Enzyme activity**,** biomarker, pesticide, liver protein, *Oncorhynchus mykiss.*

Karbosulfanın Gökkuşağı Alabalığının (*Oncorhynchus mykiss*) Eritrosit ve Karaciğer Asetilkolinesteraz (AChE) Enzim Aktivitesine Etkisi

Özet

Karbosulfanın kronik toksik etkisine maruz bırakılan gökkuşağı alabalığının (*Oncorhynchus mykiss*) eritrosit ve karaciğer asetilkolinesteraz (AChE) enzim aktivitelerindeki değişim ve enzim aktivitelerinin karbosulfana karşı hassasiyetleri araştırılmıştır. Kronik toksik test (60 gün) sonrası, balıkların enzim aktivitelerindeki normalleşmeyi belirlemek için balıklar 24 gün boyunca toksik madde içermeyen akarsuda tutulmuştur. Eritrosit ve karaciğer AChE aktivitesi, asetiltiyokolin iyotun substrat olarak kullanıldığı spektrofotometrik yöntemle ölçülmüştür. Karaciğerle karşılaştırıldığında, eritrosit AChE aktivitesinin inhibisyon oranının daha yüksek olduğu tespit edilmiştir. Maruz kalma süresiyle enzim aktivitesinin inhibisyon oranı arasında pozitif bir korelasyon olduğu belirlenmiştir. Eritrosit ve karaciğer AChE aktivitelerinin normalleşme süresi sırasıyla 18 ve 21 gün olarak belirlenmiştir. Eritrosit AChE aktivitesinin karbosulfana karşı daha hassas olmasından dolayı, bu aktivitenin sucul ekosistemdeki karbosulfan yada diğer kirleticilerin takibinde biyomarker olarak kullanılması daha uygun olabilir.

Anahtar Kelimeler: Enzim aktivitesi, biyomarker, pestisit, karaciğer proteini, *Oncorhynchus mykiss.*

Introduction

Organophosphate and carbamate pesticides act as neurotoxicants by affecting synaptic transmission in cholinergic parts of the nervous system of fishes. They are formulated to be effective inhibitors of acetylcholinesterase (AChE) through interaction on the nucleophilic active site service of the enzyme to form a phosphorylated enzyme derivative (Murthy, 1986). The severity of inhibition in the species depends on the dose, route and degree of exposure (Andreescu and Marty, 2006). AChE activity has also been used as a tool to diagnose organophosphorusand carbamate pesticide exposure in fish (Kopecka and Pempkowiak, 2004; Rendon-von Osten *et al.*, 2005; Whitehead *et al.*, 2005). Inhibition of AChE is not necessarily fatal and using a fish with a highly sensitive AChE as a sentinel species will allow the

detection of lower contamination levels of different environmental contaminants in cases of long-term and sublethal exposures (Oliveira *et al.*, 2007).

Carbosulfan [2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-n-butylaminosulfenyl) methyl carbamate], which is a carbamate insecticide, extensively used for pest control in some countries such as Mexico, Brazil, India and Sri Lanka in a wide range of crops, mainly citrus, corn, potato, soybean, and rice. The European Union banned the use of carbosulfan in 2007 (De Mel and Pathiratne, 2005; EU, 2007; FAO, 2010). Environmental concentration of carbosulfan has been ranged between 0.64 µg/L and 29 µg/L in fresh water (Leppert *et al.*, 1983; Sao *et al.*, 2008) and 0.010 mg/kg and 1.009 mg/kg in the surface soil (Acton, 2012).In the water, the metabolism of carbosulfan involves hydroxylation or oxidation reactions, or both, to be metabolized to carbofuran in animals (Giri *et al.*, 2003). Carbosulfan, as with other carbamates, is highly toxic to fish and various other aquatic organisms (Chandrasekara and Pathiratne, 2007). 96 h LC_{50} concentrations of carbosulfan in rainbow trout werebetween 180µg/L and 500 µg/L (PAN, 2013). The mechanism of carbosulfan toxicity is based on inhibition of AChE (Salte *et al.*, 1987; Hoy *et al.*, 1991).

Detection of low levels of pesticides in aquatic environments by analytical techniques alone may be difficult as most of them can fall below detection limits within a short period of time due to their relatively short half-life(Chandrasekara and Pathiratne, 2007). In vivo inhibition or induction of biomarkers can be considered as a good tool to assess the exposure and the potential effects of pesticides on living organisms (Ozmen *et al.*, 1999; Dembélè *et al.*, 2000; Sturm *et al.*, 2000; McLoughlin *et al.*, 2000; Rendon-von Osten *et al.*, 2005; Varo *et al.*,2007).Thefish plays an increasingly important role in the monitoring of water pollution because it responds with great sensitivity to changes in the aquatic environment (Hernández-Moreno, 2010). Furthermore, changing of enzyme activities is used to determine the presence of pesticides in water (Chandrasekara and Pathiratne, 2005; Varo *et al.*, 2007).

In fish, previous studies about pesticides have focused on inhibition of enzyme activities (Tridico *et al.*, 2010; Sharbidre *et al.*, 2011; Li *et al.*, 2011), with most of the studies focusing on the recovery process (Straus and Chambers, 1995; Du *et al.*, 2009). AChE activity was characterized in brain, muscle and liver (Bretaud *et al.*, 2000;Almeida *et al.*, 2005;Halappa and David, 2009; Modesto and Martinez 2010). Reports regarding comparative effects ofpesticide on the blood and liver of the fish are scanty. In the presentstudy, we aimed to determine the effects of long term (60 days) exposure to carbosulfan on erythrocyte and liver AChE activity in rainbow trout (*Oncorhynchus mykiss*), and to assess which of them was more sensitive to carbosulfan.

Materials and Methods

Pesticide

Carbosulfan (100% technical grade), a carbamate insecticide, was obtained from Sigma–Aldrich (Taufkirchen, Germany). Stock carbosulfan solutions (30 mg/L) were prepared by diluting with double-distilled water to obtain required concentrations. Stock solution concentration was kept higher than the actual concentration for elimination of carbosulfan lost in the water due to hydrolysis, photolysis, bioaccumulation and other factors.

Fish

Rainbow trout *Oncorhynchus mykiss* (116.88±21.69 g; 22.39±1.40 cm; Mean±SD) were obtained from Karadeniz Technical University, Faculty of Marine Sciences, Trabzon, Turkey. Fish were held in two flow-through tanks (200 L) at 12±1.4°C for at least 15 days to acclimatize to laboratory conditions prior to experiments.The fish were examined and determined to be free of external parasites and no deaths or disease symptoms were recorded during the acclimatization period (AFS-FHS, 2003). Dissolved oxygen concentration was 8.45±0.15 mg/L, and pH was 7.5±0.3. Throughout the acclimatization and subsequent periods of carbosulfan exposure, fish were held under a photoperiod of 12 h of light and 12 h of darkness (ILAR 1996).Twenty fish were measured to determine average weight/length before the experiment.

Water Quality

During the chronic exposure of carbosulfan and recovery test, water quality characteristics and carbosulfan concentration in each treatment were measured daily. Total ammonia was measured by an indophenol method, and nitrite was measured by an azo method. Total hardness and total alkalinity were measured by titration method. Dissolved oxygen concentration was measured by Winkler method (Boyd and Tucker 1992). Water temperature and pH were determined with a glass electrode (Thermo Orion, Beverly, MA, USA).

Exposure Conditions and Recovery Period

The experiments wererun in flow-through systemsthat wereconducted for 60 days. Based on the acute toxicity tests of carbosulfan(Boran *et al.*,2007),25 µg/L of carbosulfan concentration (11%96-h LC_{50}) was selected for chronic exposure taking into account the environmental concentrations. After acclimatization, fish were exposed to the sublethal concentration of carbosulfan in groups of 50 fish in 200 L of the test water in flow-through fiberglass tanks for 60 days. The experiments were

conducted in duplicates and control fish were also maintained in two flow-through fiberglass tanks (50 fish in 200 L in each aquarium) (US EPA, 1996). Average starting size was the same among replicates. Water flow for each tank was 6 L/h and carbosulfan test solution (30 mg/L) was added to infusion pump at the rate of 5.25 mL/h to ensure 25 ±0.16 µg/L actual concentration of carbosulfan. During the carbosulfan exposure, four fishfrom each tank (total of 8 in each group) were sampled biweekly to determine enzyme activities.

During the treatment, water in each aquarium was aerated. Fish were fed with commercial trout pellets daily at 2% body weight (BW).During the carbosulfan exposure, water temperature, oxygen, pH, ammonia and nitrite were 12.38±0.04°C, 8.30±0.12 mg/L, 7.22±0.07, 21.54±2.24 ng/L and 33.18±0.57 µg/L, respectively.Water quality was suitable for chronic toxicity test according to US EPA (1996).

At the end of the 60 days of sublethal toxicity tests, fish were transferred into flow through tanks to observe further effects of carbosulfan. Fish were allowed to recover in toxicant-free water for 24 days. During the recovery period, two fish in each group were sampled daily to determine enzyme activities. Water in each tank was aerated and water quality parameters were measured daily. Fish were fed with commercial trout pellets daily at 2% BW.

Determination of Carbosulfan in Water Samples

Carbosulfan in water samples was determined according to Sao *et al.* (2008). Briefly, 0.3 mL of 1 N $H_2SO_4$was added to an aliquot of a standard solution containing carbosulfan (8–56 µg) in a graduated flask. It was hydrolyzed in phenol by adding 0.5 mL NaOH. Then 1.5 ml diazotized p-aminoacetophenone was added and the solution was kept for 5 min with occasional shaking to ensure complete coupling. One-milliliter of 4 M NaOH was added and the volume was made up to 10 ml. Water samples were extracted twice with 5 ml chloroform in a separating funnel. Extract was evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml ethanol and diluted up to 50 ml with double distilled water. Absorbance of water samples and standard solutions were measured at 465 nm utilizing UV–VIS Spectrophotometer (Shimadzu 2550, Corporation Kyoto, Japan).

AChE Activity

Erythrocyte AChE activitywas determined in weekly for the first month, and then twice in a month. Approximately, 600 µL blood samples of were taken from fishes by caudalpuncture with a 1-ml syringe and blood was transferred into heparinized tubes containing 66 µL of 0.1 M sodium- EDTA.Blood samples were centrifuged for 1 h (4000g) at 4°C and erythrocytes were washed two times with 0.9% NaCl and then resuspended in 0.5 mL potassium phosphate (12.5 mM, pH 7.4) for enzyme assay (Gu and Chang, 2009).AChE activity was determined by the method of Ellman *et al.* (1961). This assay depends on the hydrolysis of acetylthiocholine iodide. The hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anionas a result of the reaction of 5,5-Dithiobis-(2-nitrobenzoic acid) (Sigma, St. Louis, MO, USA) with thiocholines. AChE activity was assessed at a wavelength of 412 nmutilizing UV–VIS Spectrophotometer (Shimadzu 2550). Measurements were conducted in triplicates. Activity was expressed as µmol/(min% hematocrit).Hematocrit was measured with capillary hematocrit tubes.

Liver tissues were dissected using sterile equipment and stored at −80°C until the protein and enzyme analysis. Protein content in the liver of fish was estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.Liver tissues were homogenized (1:10, w/v) in homogenization buffer [100mM KCl and 1m M EDTA (pH7.4)] at 9500 rpm for 1.5 min. Homogenates were centrifuged at 10000 g for 30 min (+4°C). Supernatants were used as an enzyme source (Atli and Canli 2010). AChE in the liver determined by the method of Ellman et al (1961). AChE activity was expressed as µmol/min/mg protein.

Statistical Analysis

Statistical analyses were performed using SPSS Version 16 software (Systat Software Inc., CA, USA). Results were expressed as means±SEM. Normality of data was checked using normal Q–Q Plots and Shapiro–Wilk test of normality. Homogeneity of variances was checked using Levene's test of homogeneity of variance. One-way ANOVA was used to determine mean differences among different times. ANOVA or Robust test of Equality of Means tables were used to determine presence of significant differences among means ($p < 0.05$). Post hoc tests were conducted using Tukey or Games–Howell tests. Significant differences were analyzed by t tests to determine which individual groups were significantly different from the control.

Result

No fish died during the carbosulfan exposure and recovery period. Treated fish showed some abnormal behavior such as darkeningcolor, loss of balance, and failure to feed after first day compared to control group. Although some improvement was seen in exposed fish afterthe second week, fish showed the same behaviors during the toxicity test.During the experiment actual concentration of carbosulfan was measured as 25±0.16 µg/L (Table 1).

The growth rate of the fish was significantly influenced by carbosulfan. Although at the beginning

of the experiment fish weight were similar in control and carbosulfan exposed fish (116.88±21.69 g), at the end of the experiment carbosulfan exposed fish weight were 120.38±18.61 (3% weight gain) while control fish weight were 164.93±9.0 (39%).

Protein levels of the rainbow trout in the liver were significantly decreased($P < 0.05$) after carbosulfanexposurewhen compared with control group (Figure 1) while the hematocrit levelof the fish was not affectedby carbosulfan (data not shown). At thebeginning of the experiment, AChE activities in the erythrocyte andliver of the carbosulfan exposed fishwere 115.79±9.10 µmol/(min% hematocrit) and 93.58±0.90µmol/min/mg protein, respectively. The erythrocyte AChE activities decreased to 68.64±5.85 mmol/(min% hematocrit) in the third weekwhile liver AChE activities decreased to 67,50±1.93 µmol/min/mg protein in the fourth weekand stayed the same until end of the experiment compared to thoseof control fish. Control group AChE activities did not change during the experiment. After one week of carbosulfan exposure,AChE activities were significantly decreased when comparedwith control fish(Table 2).

Erythrocyte AChE inhibition rate was 27.03% in the first week and 41.31% in the fourth week and then inhibition ratewere stayed the same at rest of the experiment.Inhibition rate of the liver AChE was increasedfrom 6.74% (week 1) to 26.81% (week 6).At the end of the experiment, erythrocyte and liver AChE inhibition rateswere 41.82% and 27.12%, respectively (Figure 2).

Fish were allowed to recover in toxicant-free water for 24 days after 60 days of carbosulfan exposure. The recovery was started for erythrocyte and liver AChE after 8 d and 10 d, respectively(Figure 3). The recovery was greater in erythrocyte than in liver. Erythrocyte AChE activity recovered after 18 d while it was 21 d for liver. At the end of recovery experiment, AChE activities for erythrocyte and liver were 115.0± 2.13 µmol/(min% hematocrit) and 92.50±1.70 µmol/min/mg protein, respectively.

Discussion

The pesticides that are not lethal to fish may affect their reproduction, metabolic disturbances, and growth (Kegley *et al.*, 1999). Organophosphates and carbamates are major agrochemicals that strongly affect different neuroenzymes and the growth of various fish species. Exposure to pesticides affects the

Table 1. Determination carbosulfan concentration in the water during the experiment

Time (Days)	Amount of carbosulfan (30 mg/L) added, mL/h	Amount of carbosulfan found, µg/L	Water flow rate (L/h)
1	5.25	25.14	6
2	5.25	24.85	6
5	5.25	25.21	6
10	5.25	25.32	6
20	5.25	25.33	6
30	5.25	25.13	6
40	5.25	25.20	6
50	5.25	25.11	6
60	5.25	25.21	6

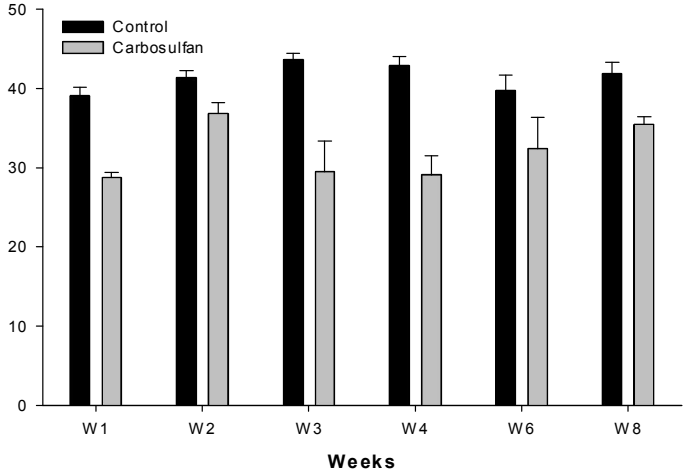

Figure 1. Protein levels in the liver of rainbow trout exposed to carbosulfan. Four fish of each group were sampled biweekly. Differences between carbosulfan-exposed fish and control fish were significant at each sampled time point ($P < 0.05$).

Table 2. AChE enzyme activities in the blood and liver of rainbow trout exposed to carbosulfan (25 µg/L)

Weeks	Erythrocyte (µmol/min % hematocrit)		Liver (µmol/min/mg protein)	
	Control	Carbosulfan-exposed	Control	Carbosulfan-exposed
1	115.79 ± 9.10	84.49 ± 5.65*	93.58 ± 0.90	91.10 ± 1.34
2	112.49 ± 5.23	75.12 ± 1.18*	91.79 ± 0.99	84.81 ± 3.93*
3	116.97 ± 5.74	68.64 ± 5.85*	95.45 ± 1.10	80.75 ± 2.40*
4	110.40 ± 9.27	65.46 ± 8.32*	89.07 ± 1.90	67.50 ± 1.93*
6	112.93 ± 8.21	67.51 ± 3.36*	88.82 ± 1.50	65.53 ± 0.98*
8	114.92 ± 7.51	66.85 ± 3.70*	93.31 ± 1.70	68.56 ± 2.70*

*: Differences between carbosulfan-exposed fish and control fish were significant (P< 0.05).

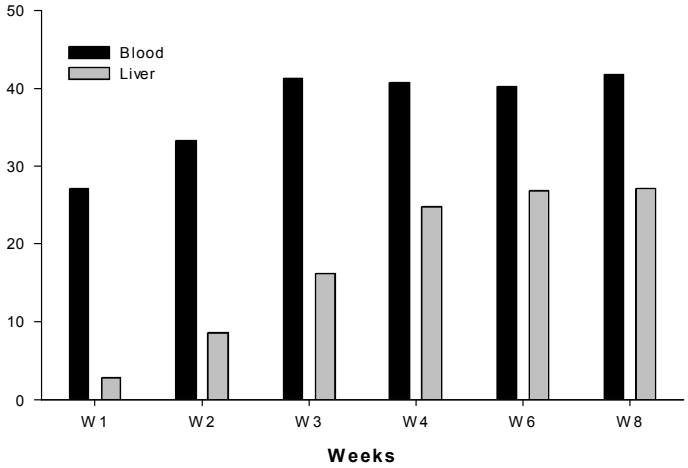

Figure 2. Inhibition rate of acetylcholinesterase (AChE) activity in blood erythrocyte and liver of the rainbow trout.

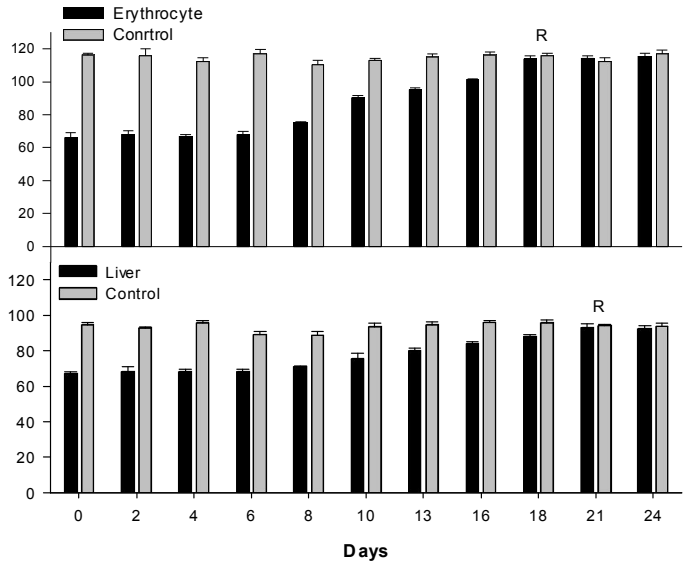

Figure 3. Recovery time (R) of AChE activity in blood erythrocyte and liver of the rainbow trout after toxicity of carbosulfan.After transferring fish to clean water fish liver and erythrocyte AChE activity was recovered after 21 d and 18 d, respectively.

functions of organs, including metabolism and neurotransmission, to various extents at different exposure concentrations (Ghazala *et al.*, 2014). Furthermore, there is a correlation between growth impairment, feeding activity and AChE inhibition (Jordaan *et al.*, 2013). Similar to these studies, in the present study, growth rate of the fish was significantly reduced by carbosulfan. Recent studies indicate that pesticides significantly decrease the total protein in the tissues (Palanikumar *et al.*, 2014; Harabawy and Ibrahim, 2014). Total protein content in different tissues of fish decreased with increasing concentration of lambda-cyhalothrin and highest decline was observed in liver protein content when compared with

muscle and brain tissues (Bibi *et al.*, 2014). Our findings agree with these studies. Liver protein content of the fish was significantly influenced by carbosulfan. It can be said that decrease in protein content in liver may be associated with metabolic degradation.

In living organism's enzyme activities and antioxidant defense systems such as AChE, catalase, superoxide dismutase and glutathione reductase are involved to counteract the toxicity of reactive oxygen species (Ellenhorn *et al.*, 1997; Orbea *et al.*, 2002). At normal states, these antioxidants protect the cells and tissues from oxidative damage. The fish from polluted sites are under oxidative stress, and the enzyme is involved in defense mechanism against peroxidative products (Orbea *et al.* 2002; Amado *et al.* 2006). It was observed that the fish exposed to environmental pollution had showed modifications in blood parameters such as deformation and change enzyme activity (Gabryelak and Pérès, 1986; Pacheco and Santos 2002). Although antioxidant enzyme inhibitory effects of pesticides were tested in fish (Kavitha and Venkateswara 2008; Altinok *et al.*, 2012), the similar effects of carbosulfan have not been compared so far in blood and liver at the same time. In the present study, AChE activities in blood and liver were studied after exposing fish to carbosulfan and it was found that AChE activities can be used as a biomarker for observation of carbosulfan contamination in natural water.AChE is responsible for the degradation of the neurotransmitter acetylcholine in cholinergic synapses, and pseudocholinesterases that are involved in the detoxification of several xenobiotics (Antó *et al.*, 2009). It is more stable and much less variable due to exposure to anti-ChE (Ellenhorn *et al.*, 1997). Erythrocyte AChE activity was found to be more sensitive to adverse effects of carbosulfan than the liver AChE activity examined in the present study. It may be more useful indicator for environmental monitoring.

For environmental risk assessment, a comprehensive understanding on adaptation and/or a recovery is essential (Du *et al.*, 2009). In the present study, after transferring carbosulfan exposed fish to clean water, erythrocyte and liver AChE activities were recovered after 18 d and 21 d, respectively. Similarly, after exposing *Gambussia affinis* to monochrotofos, AChE activity was recovered in 20 d (Kavitha and Venkateswara, 2007). AChE activity in *Salmo salar* exposed to fenitrothion was reversible after 6 weeks of recovery (Morgan *et al.*, 1990).

Blood is relatively easy to sample and many effect parameters are easy and fast to analyze. In the present study, none of the fish were killed during the blood sampling. Therewithal, blood erythrocyte AChE was more sensitive to carbosulfan than the liver AChE. Therefore, it is notable that non-lethal techniques such as blood collection from the caudal vein of fish should be used to determine potential effects of other chemicals to the surrounding environment, or it should be used for bio monitoring purposes.

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