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RESEARCH ARTICLE

Biochemical responses of a freshwater fish *Cirrhinus mrigala* exposed to tris(2-chloroethyl) phosphate (TCEP)

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Abstract

Freshwater fish *Cirrhinus mrigala* were exposed to tris(2-chloroethyl) phosphate (TCEP) with three different concentrations (0.04, 0.2, and 1 mg/L) for a period of 21 days. During the study period, thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) levels were significantly ($p < 0.05$) inhibited. The superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and lipid peroxidation (LPO) levels were increased significantly ($p < 0.05$) in gills, liver, and kidney tissues, whereas glutathione (GSH) and glutathione peroxidase (GPx) (except liver tissue) activities were inhibited when compared to the control group. Likewise, exposure to TCEP significantly ($p < 0.05$) altered the biochemical (glucose and protein) and electrolyte (sodium, potassium, and chloride) levels of fish. Light microscopic studies exhibited series of histopathological anomalies in the gills, liver, and kidney tissues. The present study reveals that TCEP at tested concentrations causes adverse effects on fish and the studied biomarkers could be used for monitoring the ecotoxicity of organophosphate esters (OPEs).

Keywords Organophosphate esters · Thyroid hormones · Oxidative stress · Ionic compounds · Histopathology · Fish

Introduction

Flame retardants (FRs) are organic chemicals that are used extensively in many polymer-based industrial and house-hold goods to prevent the chances of explosion or to avoid damage of materials during fire accident. The usage of FRs is increasing every year (Mags- Novel; News and Events 2015). The major concern is that FRs do not bind with the polymers; hence, the possibility of leaching into their environment is higher. FRs have been detected in environment including human body fluids up to micrograms or even more; unfortunately, these chemicals have potential to cause toxicological impacts on living organism even at low level (Morgan et al. 2019). Different groups of FRs such as bromine, chlorine, phosphorus, nitrogen, boron, and metallic hydroxide-based organic compounds have been utilized so far. Some of the FRs are listed as persistent organic pollutants and banned for using further (Sugeng et al. 2017; Hao et al. 2018; Hou et al. 2019). Organophosphate flame retardants (OPFRs) with logKow ranges from 1.44 to 9.49 and logKoc from 2.21 to 6.87 have been used as substitute for toxic FRs and additives in combustible products up to 15% (Cristale et al. 2016; Wolschke et al. 2018; Chokwe and Okonkwo 2019). During manufacturing, these OPFRs might leach and reach the environment. The persistence nature of their chlorine atoms in the environment could be a contributor for the formation of various hazardous polychlorinated compounds such as dibenzo-p-dioxins, dibenzofurans, and dioxin-like polychlorinated biphenyls, hexa- and penta-chlorobenzene, and penta-chlorophenol (Matsukami et al. 2014).

Among OPFRs, tris(2-chloroethyl) phosphate (TCEP) is a high production volume chemical, which is extensively used in many polymeric materials such as adhesives, polyvinyl chloride, textiles, baby products, plastics, electric and electronic products, and building materials up to 30% (Liu et al. 2016;

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Wolschke et al. 2016). Chemically, TCEP are classified as semi-volatile organic compounds that are used as an alternative for toxic polybrominated diphenyl ethers (PBDEs) (Wu et al. 2017; Chen et al. 2018). The global market demand for these compounds has been increased from 5 lakhs tones (in 2011) to 7 lakhs tones (in 2015) and the demand was expected to rise by 15% every year (van der Veen and de Boer 2012; Wang et al. 2015; Li et al. 2016). Like other FRs, their chemical nature (volatilization, leaching, and abrasion properties) increases migration capacity to reach their surrounding environment (Bollmann et al. 2012; Wei et al. 2015). As an evidence, the presence of TCEP in dust (330,000 ng/g), human excreta (2.1 ng/L), and breast milk (2.1 ng/g to 8.2 ng/g) was reported worldwide (Sundkvist et al. 2010; Ding et al. 2015; Zhang et al. 2017; Zhang et al. 2018). TCEP have low removal efficiency and thus, they are predominant contaminant among the chlorinated aliphatic esters in the aquatic ecosystem. Mean concentration of TCEP in the wastewater treatment plant in Germany was ranged up to 370 ng/L (Reemtsma et al. 2008). Highest concentration of TCEP (87.4 mg/L) was recorded in raw water from a Japanese sea-based wastewater disposal site (as mentioned in Du et al. 2019). TCEP have also been detected in various water bodies. OPFR concentration in water ranged 9.6 to 1549 ng/L, among them, TCEP accounted for > 69% with a maximum concentration (268 ng/L) and the most contaminant of Xiaoling River, China (Wang et al. 2015). TCEP have been detected up to 130 ng/L in River Ruhrare, Germany (Andresen et al. 2004), ranged up to 130 ng/L in river water of Austria (Martínez-Carballo et al. 2007), and quantified up to 61 ng/L in lake water of Germany (Regnery and Püttmann 2010). TCEP persist and tend to move between the environments; thus, it can reach the aquatic organisms (Liu et al. 2016). TCEP occurrence has been reported in biological sample collected from the Pearl River Delta region, China (Ma et al. 2013), in fishes from Great Lakes basin, USA (Guo et al. 2017), and in the collected samples such as crab, blue mussel, burbot liver, and cod liver from the regions of Norwegian Arctic (Evens et al. 2009).

TCEP exposure could cause various acute and chronic toxicological effects on organisms, such as reproductive system and fertility abnormalities in fishes (Liu et al. 2012; Ta et al. 2014; Arukwe et al. 2018), tumors in the liver and kidney of rodent (Matthews et al. 1993; Zhang et al. 2017), increases carcinogenic activity, and provokes thalamus neuronal necrosis in the mammalian models (mice and rat) (Moser et al. 2015; Xu et al. 2017). There are also reports on steroidogenic pathways in juvenile salmon (Arukwe et al. 2016; Arukwe et al. 2018), DNA damage, changes in acetylcholinesterase (AChE) activity, alteration in gene expression and oxidative stress in earthworms (Yang et al. 2018a), and genotoxic effects on zebrafish (Wu et al. 2017) under TCEP treatments. Thus, TCEP was categorized as emerging contaminants in the environment (EC 2009; ECH 2009; Liu et al. 2017).

The freshwater system plays a major role in the growth of many aquatic organisms and is known to be an ultimate sink for most of the chemicals. Aquatic organism can act as biological markers to assess the health status of the aquatic environment. Among aquatic organism, fish are used widely in toxicological study because they are highly sensitive to slight environmental alterations. Toxicity of waterborne chemicals could be assessed by using fish bioassay. The long-term toxic effects of waterborne chemicals on fish models could help in establishing standard criteria for risk assessment and safety for aquatic environment (Zhong et al. 2018). Assessment of alterations on morphological and physiological biomarkers of fish reflects the harmful effects of environmental pollutants on aquatic organisms (Austin 1998). Thus, fish biomarkers collectively provide an insight into the overall health status of the aquatic organisms and act as an indicator of the environmental pollution status.

In fish, growth, maturation (Walpita et al. 2009), osmoregulation (Peter 2011), smoltification (Björnsson et al. 2011), and larval metamorphosis (Taillebois et al. 2011) are controlled by the thyroid metabolism. Ecological contaminations also affect the thyroid system and directly block THs (thyroid hormone) synthesis, TH-blood transport, and TH metabolism (Boas et al. 2006). Hormonal assay is a valid technique in the field of toxicology; the hormonal activity is used as an early warning signal (Folmar 1993; Hontela et al. 1993). Moreover, responses of thyroid hormones have been used in toxicological research to understand the growth and metabolism of fish under stress conditions. Previous study indicates that aquatic pollutants alter the level of thyroid hormones, which results in impairment of development and function of thyroid hormones (He et al. 2012; Katuli et al. 2014; Shirdel et al. 2016). FR once reaches the digestive system, it readily enters the circulatory system and accumulates at tissues and acts as potential thyroid hormone disruptors (Zhang et al. 2016; Curran et al. 2017). Generally, organophosphorus esters (OPEs) cause disturbances in thyroid hormone signaling and endocrine disruption action on organisms (Kojima et al. 2013; Greaves and Letcher 2017). However, disruptions in thyroid function by TCEP exposure on fish are not reported.

During metabolic reaction, body produces free radicals that could cause harmful effects. The body itself generates antioxidant to balance the free radicals. Thus, antioxidant enzyme activities are considered an important tool in toxicity studies as their levels reflect the toxicity, concentration, and exposure duration (Pamanji et al. 2016). Numerous studies explain that the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH)-dependent enzymes (e.g., glutathione S-transferases (GST), glutathione reductase (GR), glutathione peroxidase (GSH-Px), and lipid peroxidation (LPO)) are considered as a valuable biomarker to evaluate chemical-related stress in fishes. Their imbalance will

result in tissue damage and alteration in normal homeostasis in an organism (Poopal et al. 2017; Sahu et al. 2018).

Histopathology is an important tool for studying the effects of waterborne chemicals in major organs such as gill, liver, and kidney that are responsible for respiration, metabolism, and excretion in fishes, respectively (Nagarjuna and Mohan 2017; Ramesh et al. 2018). Histological anomalies are indicator for the loss of function (Fanta et al. 2003) and act as a signal of health status of an organism (Hinton and Lauren 1990).

The plasma biomarkers (e.g., glucose, proteins) are widely studied to understand the effects of xenobiotic exposures on organisms. Plasma biomarkers give the relevant data in determining the extent of cell, tissues, and organ damages and provide an early warning signal to animals in concern (Canli and Canli 2015). In addition to the above biomarkers, the measure of ionic levels such as potassium (K^+), chloride (Cl^-), and sodium (Na^+) in the blood of aquatic organisms also aids in monitoring the polluted aquatic ecosystem (Mayer et al. 1992; Sathya et al. 2012; Hemalatha et al. 2016).

Previous literatures report the occurrence, accumulation, and distribution of TCEP in various aquatic organisms (Hou et al. 2017; Arukwe et al. 2018). However, reports on toxicological responses such as primary stress indicator (hormones), oxidative stress, pathological structures, blood biomarkers (glucose and protein), and electrolyte levels with respect to FRs especially on TCEP exposures on freshwater fish are limited. Therefore, this study was undertaken to examine the responses of various biomarkers in a freshwater fish *Cirrhinus mrigala* exposed (chronic) to different concentrations (0.04, 0.2, and 1 mg/L) of TCEP. The findings of the present study might provide better knowledge on TCEP toxicity on aquatic organisms and contribute to a greater understanding of overall organophosphorus flame retardant toxicity.

Materials and methods

All the laboratory analyses and fish maintenance were performed by following the guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Fish

Freshwater fish (*C. mrigala*) with an average length 7.0 ± 0.1 cm and weight 12 ± 0.1 g were obtained from Aliyar fish farm, Pollachi, Tamil Nadu, India, and housed at Toxicology Lab, Department of Zoology, Bharathiar University, Coimbatore, Tamil Nadu, India. The fingerlings were adapted to laboratory conditions for 2 weeks in a tank containing dechlorinated tap water with temperature 26 ± 0.1 °C, pH 7.2, dissolved oxygen 6.6 ± 0.02 mg/L, and 12:12-h photoperiods and used as main stock. During the acclimatization periods, rice bran and groundnut oil cakes were fed (once in a day) in the form of dough. The water in the tank was renewed every 24 h to make sure the tank is free from fecal matter and enough oxygen is supplied to fish.

Test chemical

Tris(2-chloroethyl) phosphate was obtained from Sigma-Aldrich (purity 97%, CAS no. 115-96-8). Stock solution was prepared by dissolving 1 g/L of tris(2-chloroethyl) phosphate in Milli-Q water. Fresh stock solutions were used.

Experimental design

For toxicity study, healthy fingerlings were collected randomly from the main stock and kept in a separate rectangular glass aquarium (120 × 80 × 40 cm) and used as test stock. The fish in test stock were not fed for 24 h prior to the commencement of experiment. For the study, three different concentrations of TCEP (treatment-I, 0.04; treatment-II, 0.2; treatment-III, 1 mg/L) were selected based on the previous literature (Arukwe et al. 2016). A 0.04 mg/L of TCEP was mixed in a glass aquarium containing 90 L of water and 40 fish. Similar setup was made for 0.2 and 1 mg/L concentrations of TCEP and a common control (without adding TCEP) were maintained. During the exposure periods, fingerlings were fed *ad libitum*. Debris (uneaten feed and fecal matters) was removed and water in the aquarium was renewed daily by removing three-fourth of the water. Three replicates were maintained for control and TCEP-treated groups with similar setup.

Sampling frequency and collection of blood and tissue samples

Samplings were done at every 7 days until 21 days. At the end of every stipulated period, 30 fish were euthanized respectively from each group, and blood was collected by cardiac puncture, then centrifuged at 10,000 rpm for 10 min and the plasma was used for the hormonal assay (thyroid-stimulating hormone [TSH], triiodothyronine [T3], and thyroxine [T4]), electrolytes (Na^+, K^+, and Cl^-), and biochemical (glucose and protein) parameters. Simultaneously, tissues such as gills, liver, and kidney were homogenized (Teflon-coated mechanical tissue homogenizer) with phosphate buffer (pH 7.4) in ice cold conditions, then centrifuged at 5000 rpm for 20 min and the supernatant was used for the analysis of antioxidant enzyme activity. A portion of the collected tissues were fixed in Blouin's fluid for histopathological studies.

Thyroid hormone assay

TSH, T3, and T4 levels in control and TCEP-treated fingerlings were measured by following the manual provided by the TOSOH commercial ELISA kits (product code: 8025-300D) (Hoseini et al. 2014). The optical density (OD) of the samples was measured by using an automatic ELISA plate analyzer (Avecina Touch Plate Reader, Iran) at 450 nm and expressed as nanograms per milliliter (ng/mL).

Antioxidants assay (gills, liver, and kidney)

SOD activity in gills, liver, and kidney of the fish was determined by the method of Marklund and Marklund (1974) and the activity was expressed as U/mg protein. CAT activity was assayed following the method of Aebi (1984) and the activity was expressed as μmol H_2O_2 utilized/min/mg protein. GST activity was determined by the method of Habig et al. (1974) and the enzyme activity was expressed as μmol GSH-cDNB conjugate formed/min/mg protein. GPx activity was measured by following the method described in Rotruck et al. (1973) and the enzyme activity was expressed as μmol GSH oxidized/min/mg protein. The reduced glutathione levels were determined using the modified method of Ellman (1959) and expressed as micrograms of GSH formed/min/mg protein. LPO was estimated through thiobarbituric acid (TBA) assay following the method of Devasagayam and Tarachand (1987) using malondialdehyde (MDA) in the samples. LPO level was expressed as moles of MDA formed/mg protein. All the assays were carried out using UV-visible spectrophotometer (Jasco, V-530).

Glucose and protein assay

Glucose level in control and TCEP-treated fingerlings was estimated using the O-toluidine method of Cooper and Mc Daniel (1970) and their OD measured against the blank at 630 nm within 30 min using a UV spectrophotometer and expressed as mg/100 mL. Plasma protein was estimated following the method of Lowry et al. (1951). The OD was read after 15 min at 720 nm using a UV spectrophotometer. A standard was also prepared, and the protein level was expressed as micrograms per milliliter.

Electrolytes assay

Na^+, K^+, and Cl^- levels were estimated using standard kit manufactured from Coral Clinical systems and supplied by Tulip Diagnostics (P) Limited, Goa, India. Sodium was estimated using the method of Maruna (1958) and Trinder (1951). Potassium was estimated following the method of Terri and Sesin (1958) and Sunderman and Sunderman (1959). Chloride was estimated by the modified method of Schales and Schales (1941) and Schoenfeld and Lewellen (1964). The levels of Na^+, K^+, and Cl^- in plasma of the fish were expressed as millimoles per liter.

Histopathological analysis

Dissected tissues (gills, liver, and kidney) were fixed in Blouin's fluid for 2 days, dehydrated with graded ethanol, infiltrated using xylene, embedded in paraffin wax, then sectioned and mounted on clean glass slides, and ended with hematoxylin and eosin staining. The tissues were examined using light microscope with image analysis system connected to a computer.

Statistical analysis

The results of the study are interpreted as mean ± S.E. The significance of the samples between control and TCEP treatments was evaluated by one-way ANOVA (analysis of variance) followed by Duncan multiple range test (DMRT). Different alphabets represent significance levels at $p < 0.05$.

Results

When fingerlings exposed to TCEP at different concentrations, behavior changes such as fast swimming, movement around the wall of the tank, and inability to feed were observed and finally settled at the bottom. In the control group, no such behavior changes were noticed. No alteration in the schooling behavior was observed (direct observation). Thus, it reveals that fingerlings used in our study are healthy.

Effects of TCEP on thyroid hormones

Plasma thyroid hormone levels in the fish exposed to TCEP concentrations were illustrated in Fig. 1a–c (TSH (a), T4 (b), T3 (c)). When compared to the control group, TSH, T4, and T3 levels were found to be decreased significantly ($p < 0.05$) in fish exposed to TCEP treatments. Among the treatment groups, thyroid hormone was declined higher at treatment III (1 mg/L). In 0.04 and 0.2 mg/L, the changes in TSH, T4, and T3 levels were found to be decreased with less significance than the control group. A steady decline was noticed in the thyroid hormone levels; among the thyroid hormones, a higher decline was observed in T3.

Effects of TCEP on gills, liver, and kidney antioxidant parameters

SOD (Fig. 2a–c) and CAT (Fig. 3a–c) activity in gills, liver, and kidney tissues was found to be increased significantly ($p < 0.05$). A steady increase in the SOD and CAT activity was

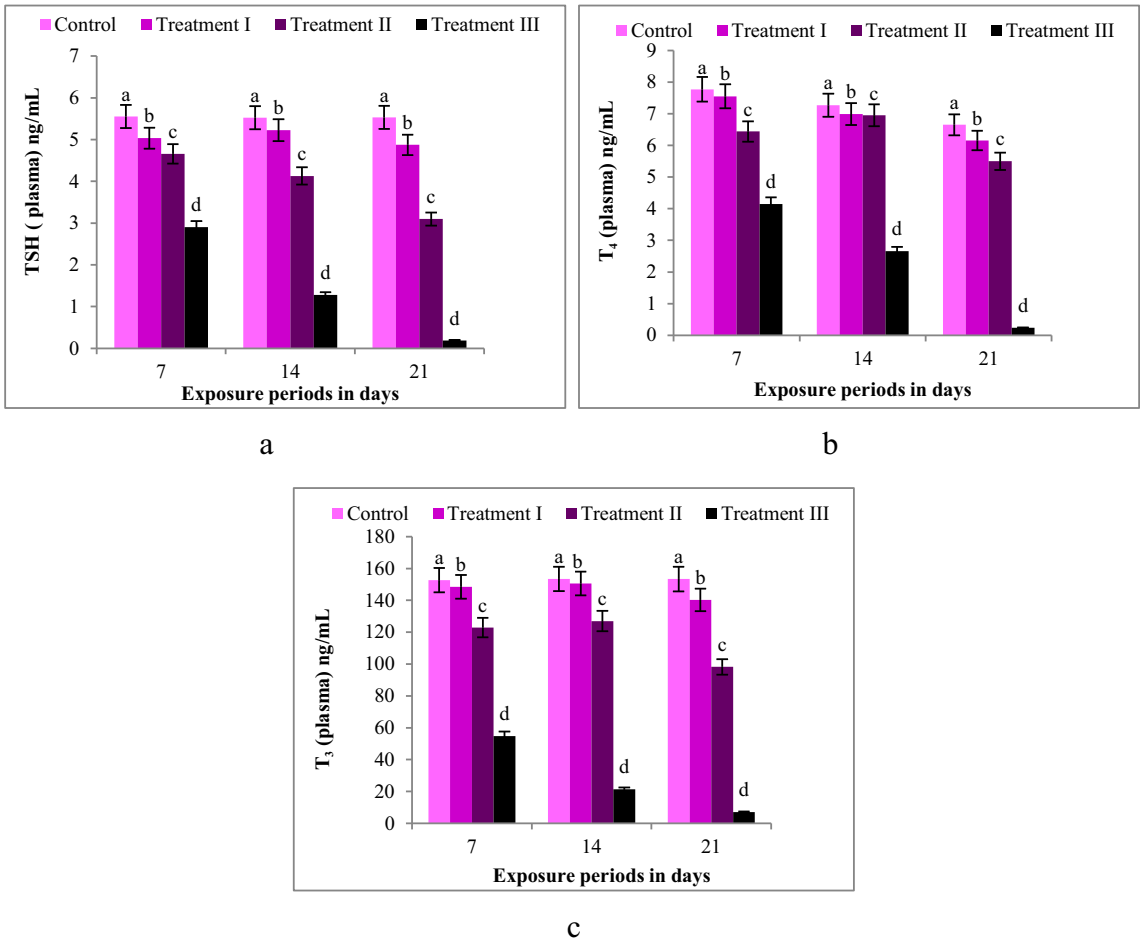

Fig. 1 Plasma TSH (**a**), T_4 (**b**), and T_3 (**c**) level of *Cirrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

observed in TCEP treatment-III group. Among the studied tissues, SOD and CAT activity was found higher in liver tissue at the end TCEP (treatment-III) exposure periods. The activity of GPx (Fig. 4a–c) in gills and kidney tissues was declined gradually, whereas in the liver tissue, the activity was found to be increased constantly when compared to the control group. The alterations were statically significant ($p < 0.05$). GSH activity in studied tissues were inhibited significantly ($p < 0.05$) in all the TCEP treatments (Fig. 5a–c). Among the tissues, liver tissue was affected higher. The GST activity (Fig. 6a–c) and LPO level (Fig. 7a–c) were elevated significantly ($p < 0.05$) throughout the TCEP exposure periods, showing a gradual increase towards the end of the study periods in the treatment-III group. Overall, a concentration-based effect was noticed in TCEP treatment groups on antioxidant parameters.

Effects of TCEP on biochemical parameters

Glucose and protein levels in plasma of fish exposed to TCEP treatments were significantly ($p < 0.05$) altered during the study period. TCEP toxicity resulted in hyperglycemic condition in *C. mrigala* (Fig. 8a). Among the TCEP treatments, higher level of plasma glucose was measured in treatment III. Protein level in plasma of TCEP-treated fish was found to be declined significantly ($p < 0.05$) throughout the study period when compared to the control group (Fig. 8b). The biochemical parameters also reveal that TCEP has duration and concentration-dependent effects on fish.

Effects of TCEP on plasma electrolytes

Plasma electrolyte levels of fish exposed to different concentrations of TCEP were illustrated in Fig. 9 (a, Na^+; b, K^+; c, Cl^-). TCEP could influence the electrolyte levels in plasma, and the alterations were found statistically significant ($p < 0.05$). In higher concentration (1 mg/L), maximum decrease in plasma Na^+ and Cl^- was observed when compared to the control group, whereas plasma K^+ level in TCEP-exposed fish was found to be increased. Maximum alterations in plasma electrolytes were noticed in TCEP treatment-III group.

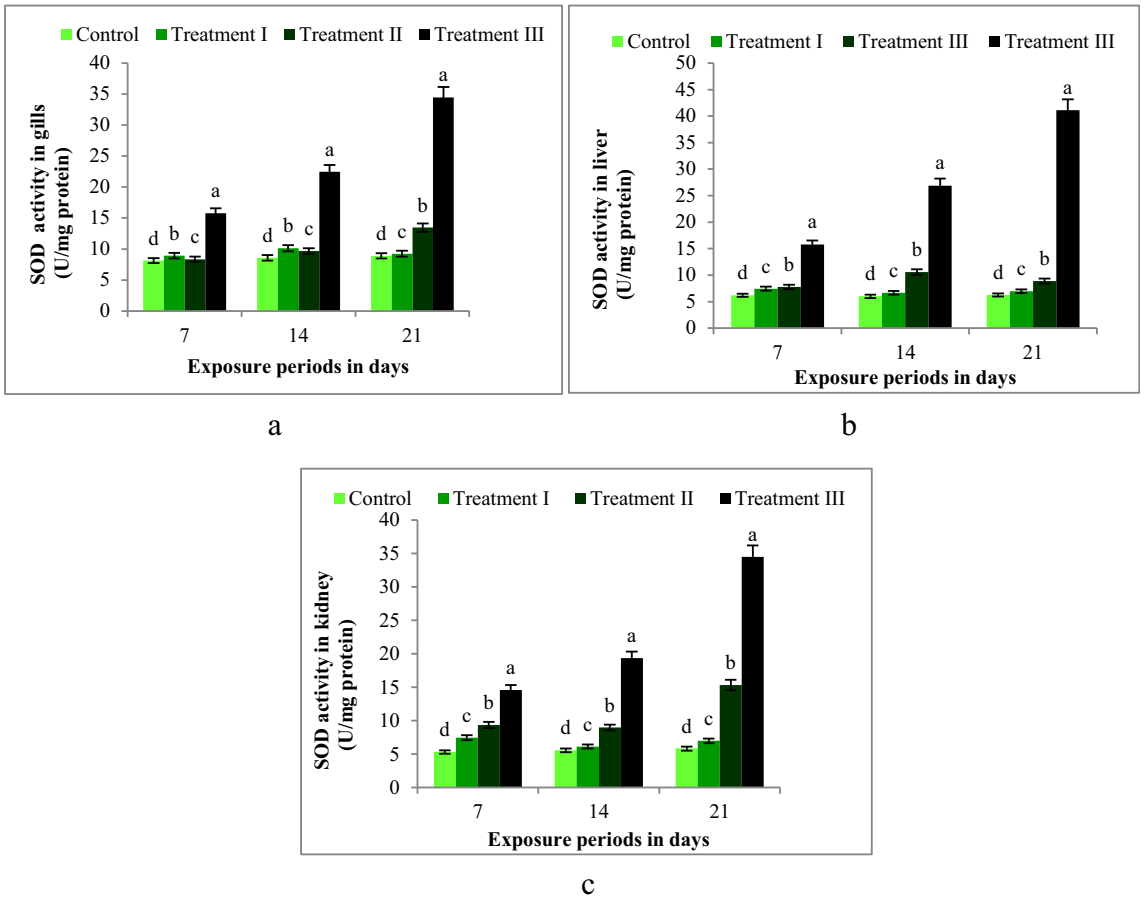

Fig. 2 SOD activity in the gills (**a**), liver (**b**), and kidney (**c**) tissues of *Cirrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

In this study, TCEP induced a concentration-based effect on electrolyte levels.

Histopathology examination

No visible structural abnormalities (gills, liver, and kidney tissues) were examined in the control group (Figs. 10a, 11a, and 12a). TCEP exposure caused epithelial lifting, hyperplasia, degeneration of cells in primary lamellae, lamellar fusion, mucus accumulation, and vacuolation in fish gills tissue (Fig. 10b–d). In liver tissue, pyknotic nuclei, fat deposition, necrosis, increased sinusoids vessels, congestion, and vacuolation were noticed in TCEP treatment groups (Fig. 11b–d). TCEP toxicity also caused hypertrophy in epithelial cells of renal tubules, shrinkage of glomeruli, expansion of Bowman's capsule, and tubular degeneration in kidney tissues (Fig. 12b–d). TCEP toxic effects on gills, liver, and kidney tissue morphology were in the series of treatment I < treatment II < treatment III. More structural anomalies related to TCEP toxicity were examined in gills and liver tissues than in kidney tissue (Tables 1, 2, and 3). TCEP caused concentration-based structural alterations in selected tissues of *C. mrigala*.

Discussion

Due to the higher and frequent utilization of TCEP, it occurs ubiquitously in the water bodies. The reported concentration of TCEP in the water system (including wastewater system) ranges several tens of micrograms per liter and a few thousands of nanograms per liter. Arukwe et al. (2016) reported that TCEP have potential to cause toxic effects on fish at mg/L levels (0.04, 0.2, and 1 mg/L). TCEP could also cause alterations on steroidogenesis or estrogen metabolism in H295R cells at different dose (0.001–10 mg/L) (as mentioned in Du et al. 2019). The data on effects of TCEP on aquatic organism are lacking (Fisk et al. 2003; Cristale et al. 2013). Thus, toxicological profiles of TCEP on aquatic organisms are much warranted (Jin et al. 2013). Therefore, to increase the availability of eco-toxicity data of TCEP, we selected different concentrations (0.04, 0.2, and 1 mg/L) and examined the potential responses of hormonal, antioxidants, biochemical, electrolyte, and histological effects on fish.

The change in the level of thyroid hormones indicates the primary stress responses of the organism exposed to toxicants. In the present study, TCEP have potential to reduce the TSH,

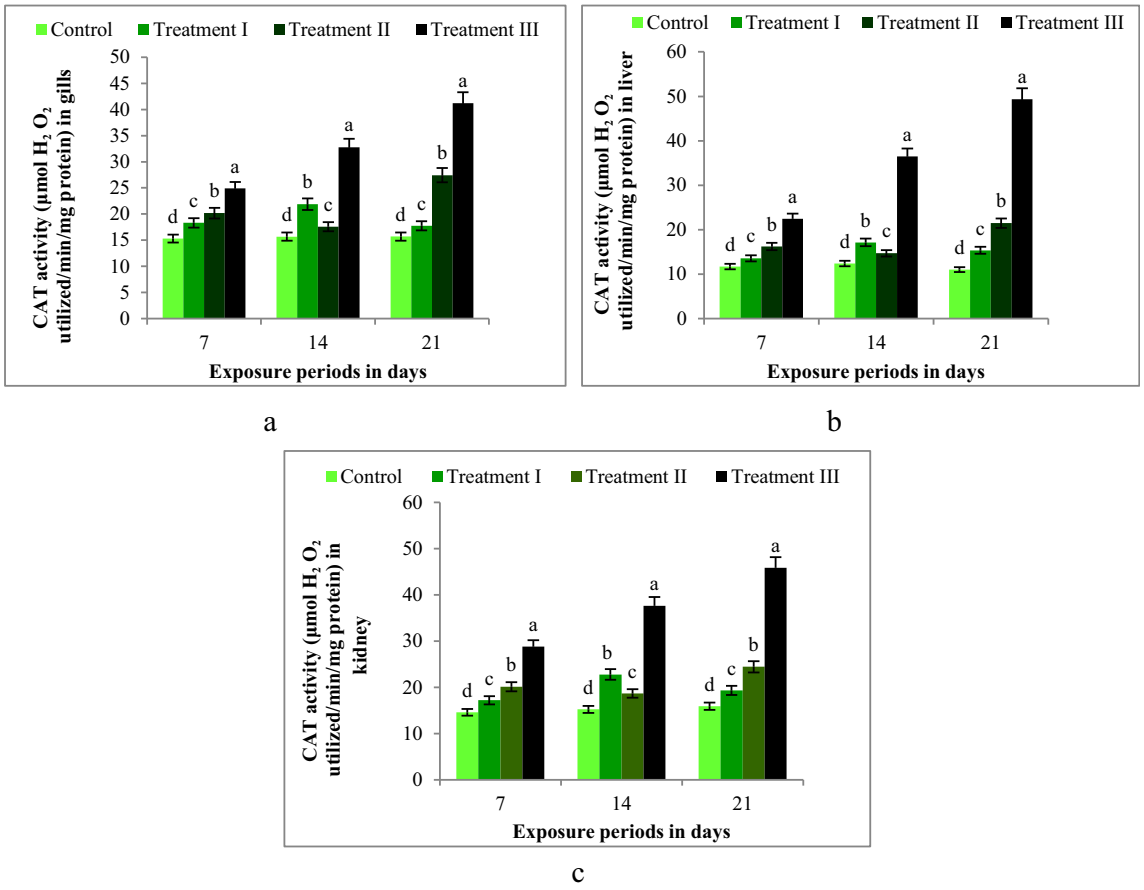

Fig. 3 CAT activity in the gills (**a**), liver (**b**), and kidney (**c**) tissues of *Cirrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

T3, and T4 levels in the plasma of the fish. Generally, thyroid hormone homoeostasis is maintained through TSH and negative feedback mechanism. The secretion and release of TSH stimulated in the hypothalamus, and TSH stimulates T4 secretion in the thyroid. T4 converted to form T3 a biologically active form (Sabir et al. 2019). Generally, the level of T4 is higher than that of T3 in the blood. The thyroid hormone levels were declined steadily as exposure period increases. This indicates that TSH synthesis was inhibited under long-term TCEP exposure either interfering directly on the structural changes in the thyroid gland, or target on the metabolism, or due to binding with protein molecules in the thyroid hormone. Inhibition in the TSH synthesis could reduce the production, conversion, and release of T4 and T3 in the plasma. Decrease in TSH levels in the blood could be a result of negative regulation at thyroidal axis or disturbance in the synthesis and release of hormone from the thyrotrope cells of pituitary (Raibeemol and Chitra 2020). The activity of UDP-glucuronosyl transferase that are responsible for glucuronidation and clearance of thyroid hormone (T4) could decrease level of thyroid hormone (T4) (Movahedinia et al. 2018). Flame retardants are known to form glucuronide metabolite through UDP-glucuronosyl transferases (Eng et al. 2019). Flame retardants are potential to cause changes in *dio* (deiodinase) gene transcription that an important enzyme for circulating and peripheral TH levels in the fish (Liu et al. 2019). Dio2 enzyme catalyzes T4 to T3 (Xu et al. 2018). Thus, the disturbance in conversion of T4 to T3 might reduce the T3 levels in the plasma of TCEP-treated groups. A significant decrease in thyroid hormone levels has been reported in *Danio rerio* exposed to tris(1,3-dichloro-2-propyl)phosphate (Xu et al. 2015). Likewise, a significant decrease in thyroxine (T4) level was reported in zebrafish exposed to tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) and tris(1,3-dichloroisopropyl)phosphate (TDCPP) (Wang et al. 2013; Kim et al. 2015) which indicates these compounds might be potential endocrine disruptors (Zhang et al. 2016). TDCIPP may alter the gene transcriptions which are responsible for the production of growth hormones (Wang et al. 2013). Tris(2-butoxyethyl)phosphate (TBOEP) altered the whole body T3 and T4 concentrations in zebrafish larvae indicating the disruptive action of the TBOEP (Liu et al. 2017). FRs could displace thyroid hormone competitively from a thyroid-binding protein, transthyretin. The displacement of thyroid hormone from its binding site could result in metabolism and elimination of hormone which results in decline of thyroid

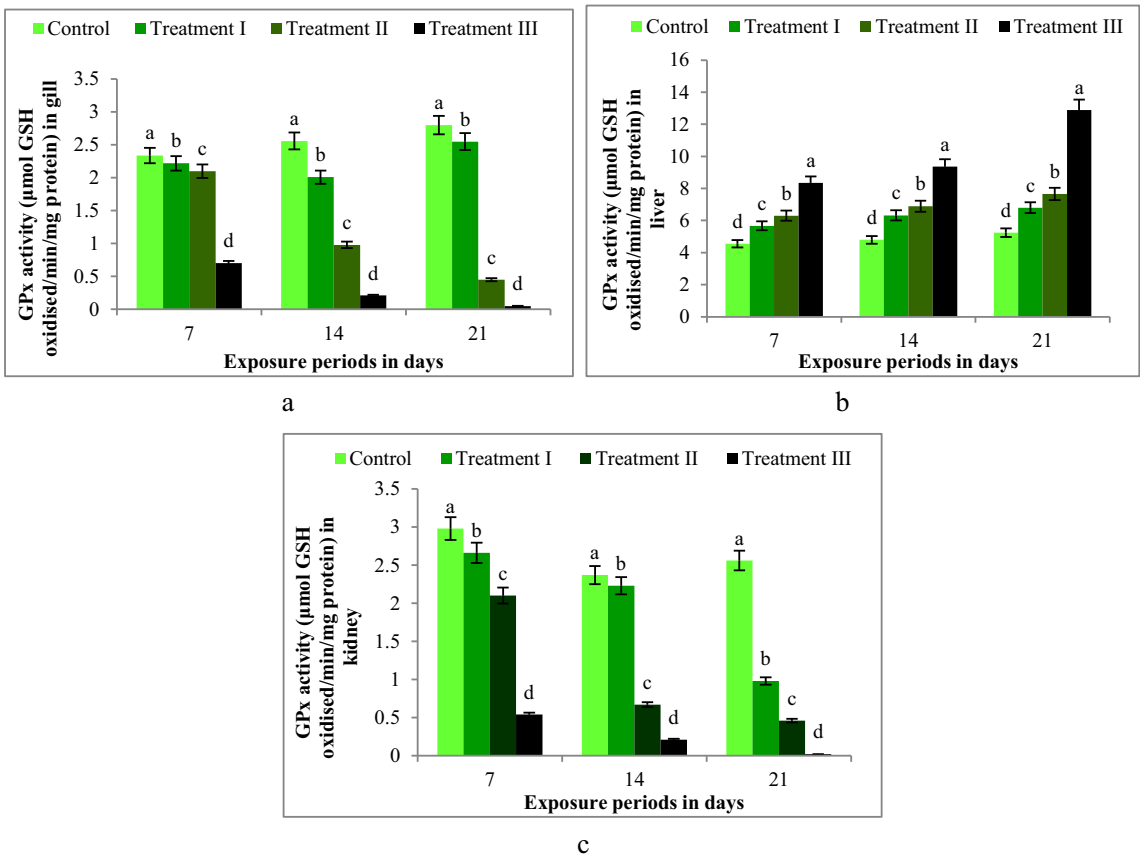

Fig. 4 GPx activity in the gills (**a**), liver (**b**), and kidney (**c**) tissues of C*irrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

hormone for circulation (Osimitz et al. 2016). From our result, we conclude that FRs could cause alterations in thyroid hormones that might affect growth and metabolism of an organism. The alterations of these hormones indicate the disruptive action of TCEP. However, the mode of action on thyroid function or the endocrine disruption potential of TCEP on aquatic organisms needs to be studied (Wu et al. 2017).

Detoxification, excretion, and balanced antioxidants could strengthen immune system to adapt aquatic organisms to different types of waterborne contaminants (Sahu et al. 2018). Generation of reactive oxygen species associates with many health defects including neurological defects, and FRs known to generate reactive oxygen species (Jarosiewicz et al. 2019). Any imbalance occurred between free radicals and antioxidants results in oxidative stress in an organism (Tabassum et al. 2016; Poopal et al. 2017). Among antioxidants, SOD, CAT, and GPx act as a first-line defense system in converting free radicals to hydrogen peroxide, molecular oxygen, and water. Their imbalance could be an indication of oxidative stress caused by stressors in an organism (Yan et al. 2017; Yonar et al. 2014). In the present study, the increased activity of SOD and CAT in gills, liver, and kidney tissues of fish indicates that the body triggered its defense mechanism against the production of free radicals during the detoxification of TCEP.

Arukwe et al. reported that bis(2-chloroethyl)carboxymethyl-phosphate, bis(2-chloroethyl)hydrogen-phosphate, and bis(2-chloroethyl)-2-hydro-xyethyl-phosphate glucuronides were the urinary metabolites of TCEP involved in the metabolic pathways such as oxidative and hydrolytic reactions, as well as glucuronidation through phase-II metabolism (Arukwe et al. 2018). The increase in CAT activity indicates the role of CAT in elimination of H_2O_2 produced by SOD (Guptha et al. 2016). In contrast to the present study, failure of antioxidant system has been reported in fish exposed to brominated flame retardant (BFR) (Feng et al. 2013) indicating that the capacity of mitigating and scavenging of free radicals by the antioxidants in the organism is governed by the toxic strength of the chemical.

GPx metabolizes H_2O_2 and reduces fatty acid peroxides. This enzyme can act on a variety of organic peroxides and catalyzes the oxidation of reduced glutathione to glutathione disulfide (Di Giullio and Hinton 2008; Plhalova et al. 2014). In our study, the activity of GPx in gills and kidney tissues of TCEP-exposed fish showed a significant decrease, whereas it was found to be increased in the liver tissue. The significant increase in

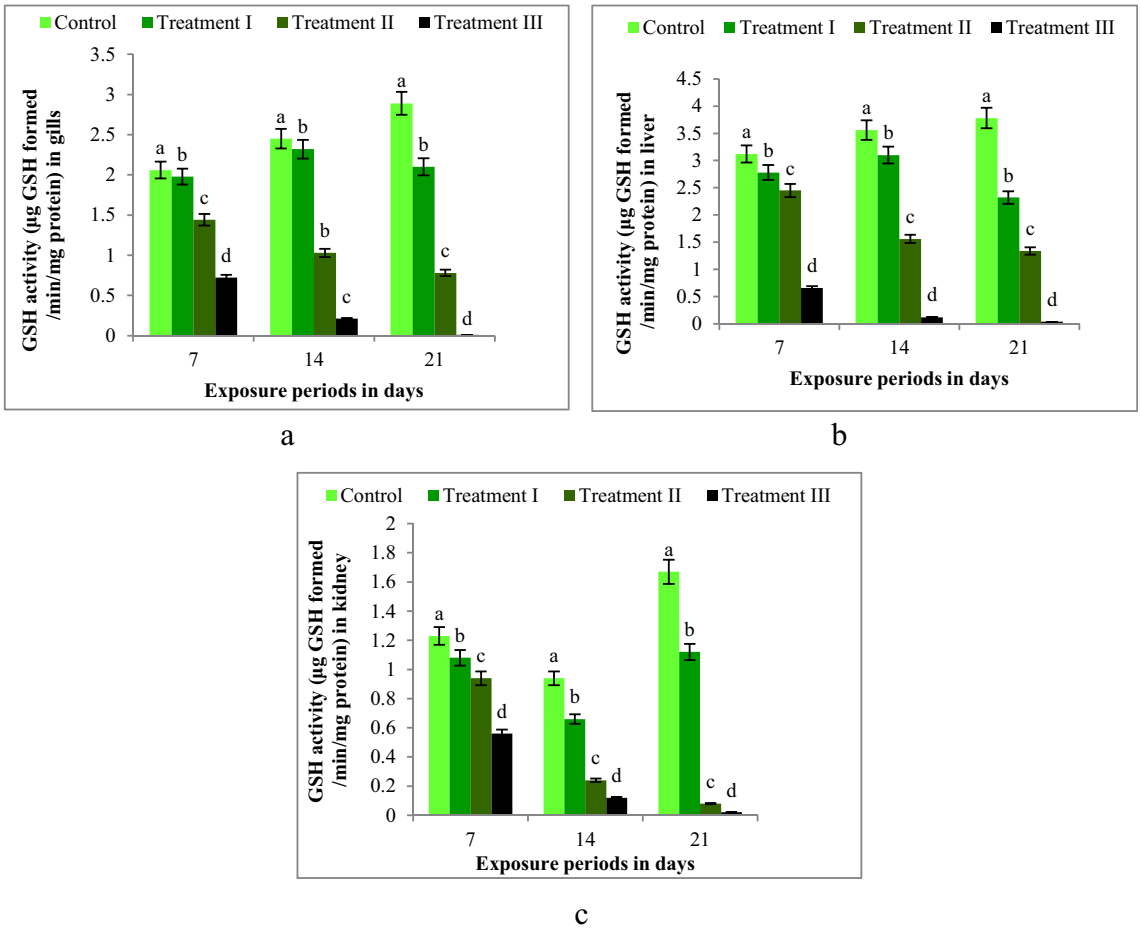

Fig. 5 GSH activity in the gills (**a**), liver (**b**), and kidney (**c**) tissues of *Cirrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

GPx activity in the liver tissue of fish indicates the activation of defense mechanism or an adaptation of fish to respond the TCEP-related ROS production (John et al. 2001; Li et al. 2009, 2010). In contrast, the decrease in GPx activity in gill and kidney tissues indicates reduced capacity to scavenge hydrogen peroxide produced in these organ/tissues (Melekh et al. 2017). Interference of TCEP on the synthesis of GPx in tissues might be another reason for the disruption of the enzyme activity. Furthermore, differences in the response of GPx activity in gills, liver, and kidney tissues might result from different mechanisms of TCEP and H_2O_2 and distribution of enzymes in tissue/organs as suggested by Tkachenko et al. (2014).

GSH is a major antioxidant that has a critical role as a co-substrate of GST in phase II biotransformation. It involves in reactive intermediates and free oxide detoxification mechanism (Samanta et al. 2014). The decrease in GSH activity in tissues is an indication of counteract to oxidative stress caused by TCEP, which is due to the imbalance of free radicals and antioxidants resulting in reduction of GSH in fish (Yonar et al. 2014). It has been reported that BFRs could induce oxidative stress, which results in increased utilization of GSH for the detoxification processes (Feng et al. 2013). GST could involve in the protective mechanism against many chemical substances (Bastos et al. 2013). In the present study, the increased activity of GST in tissues of fish is probably due to the action of defensive mechanism against the TCEP toxicity. The increase in the process of biotransformation of chemicals could activate GST in an organism (Modesto and Martinez 2010). The activity of GSH lays antiparallel to GST, i.e., inhibition of GSH results due to their exhaustion in the phase II biotransformation process with an elevation of GST activity (Kaur and Jindal 2017). Our results are in strong agreement with the authors' statement.

LPO levels could be used as an indication of health status of cell and tissues. Imbalanced antioxidant and free radical could result in alteration of LPO level (Pan et al. 2006). In the present study, we found elevation of LPO level in tissues of TCEP-exposed fish. The elevation of LPO level is an evidence of improper functioning of cells due to oxidative stress caused by TCEP (Monteiro et al. 2006; Feng et al. 2013). Likewise, tris(2-butoxyethyl)phosphate (TBOEP) at 2500

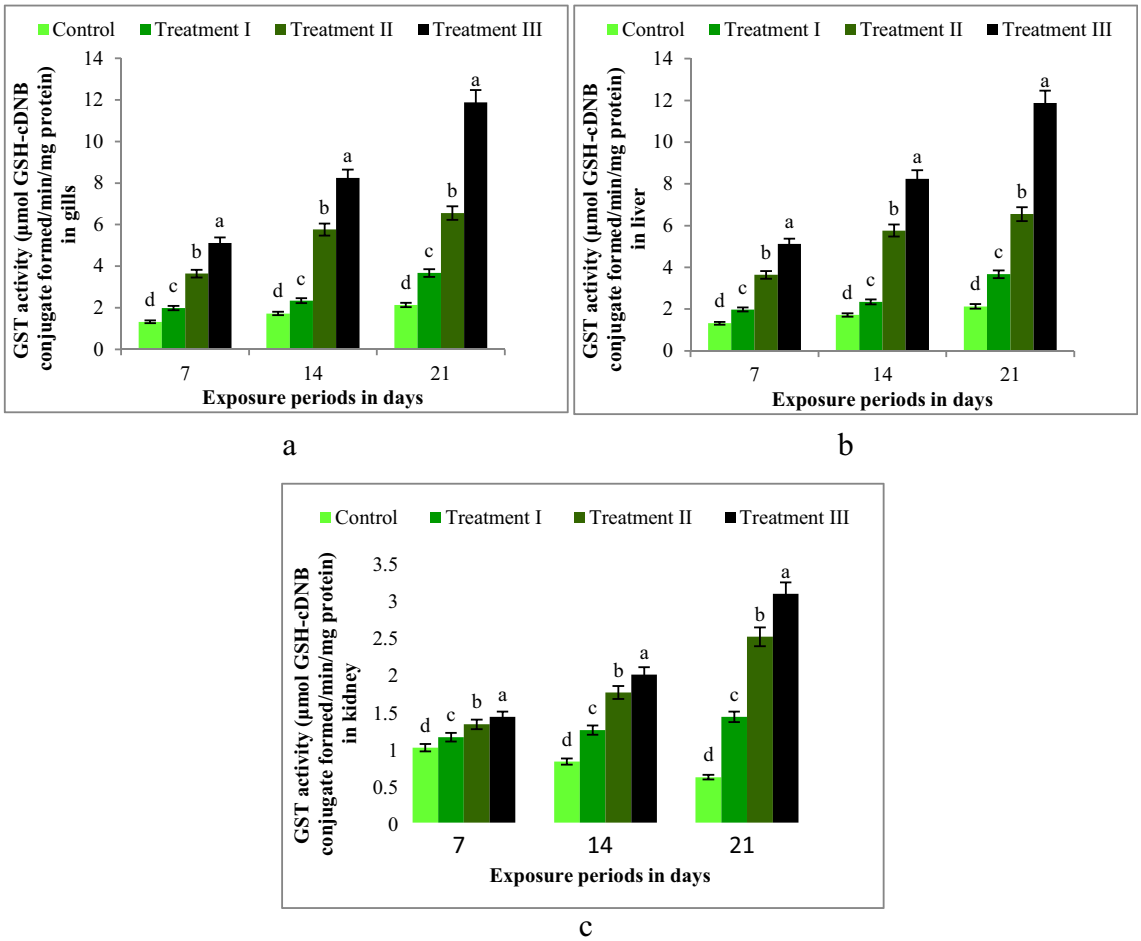

Fig. 6 GST activity in the gills (**a**), liver (**b**), and kidney (**c**) tissues of C*irrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

µg/L concentration increased the MDA level and resulted oxidative stress in zebrafish (Jiang et al. 2018). Generally, lipid peroxidation formation will be prevented by SOD activity. In our study, both LPO and SOD activities are elevated; this reveals that antioxidants are intolerable to TCEP-mediated free radicals. FRs could generate higher intercellular reactive oxygen species and damage DNA as concentration increases (Yuan et al. 2019). This reveals that TCEP have potential to generate free radicals and act on lipid profiles.

Glucose level is frequently used as stress response biomarker in toxicology. Fish exposed to TCEP showed hyperglycemic response. Generally, this situation occurs as a result of high energy demand in an organism. TCEP showed increased glucose level at higher concentration and neurological metabolic disorders on experimental mammalian model (Yang et al. 2018b). Thus, the hyperglycemic condition in the present study reveals that the fish utilize glucose to compensate stress caused due to TCEP toxicity. Proteins have significant role in many metabolic actions in the body and their concentrations are widely used as health indicator in the field of toxicology. Proteins are utilized as major among the energy sources under stress conditions (Woo et al. 2018). Hypoproteinemia in the TCEP-exposed fish indicates the catabolism of protein to meet the energy demand generated during elimination or detoxification process. The protein might act as a compensatory action for inefficiency of immune action to tolerate TCEP-mediated stress. Moreover, TCEP may influence citrate cycle by elevating citrate and 2-oxoglutarate which result in energy deficiency (Deng et al. 2018); this could result in gluconeogenesis process, which ultimately alter biochemical (glucose and protein) levels to meet out the energy demand caused in the fish exposed to TCEP.

Electrolyte imbalances are important biomarkers that have been widely studied in toxicity assessment (Ajima et al. 2018). Levels of Na^+, K^+, and Cl^- in circulatory system are widely used to assess the health conditions of aquatic organisms (Poopal et al. 2013; Hemalatha et al. 2016). Na^+ and Cl^- level in the plasma of TCEP-exposed fish was declined. The K^+ level was found higher in TCEP-treated fish. In the present study, accumulation of TCEP in gills and liver may lead to osmoregulatory failure which results in alterations of plasma ions. Accumulation of TCEP has been reported in fish species

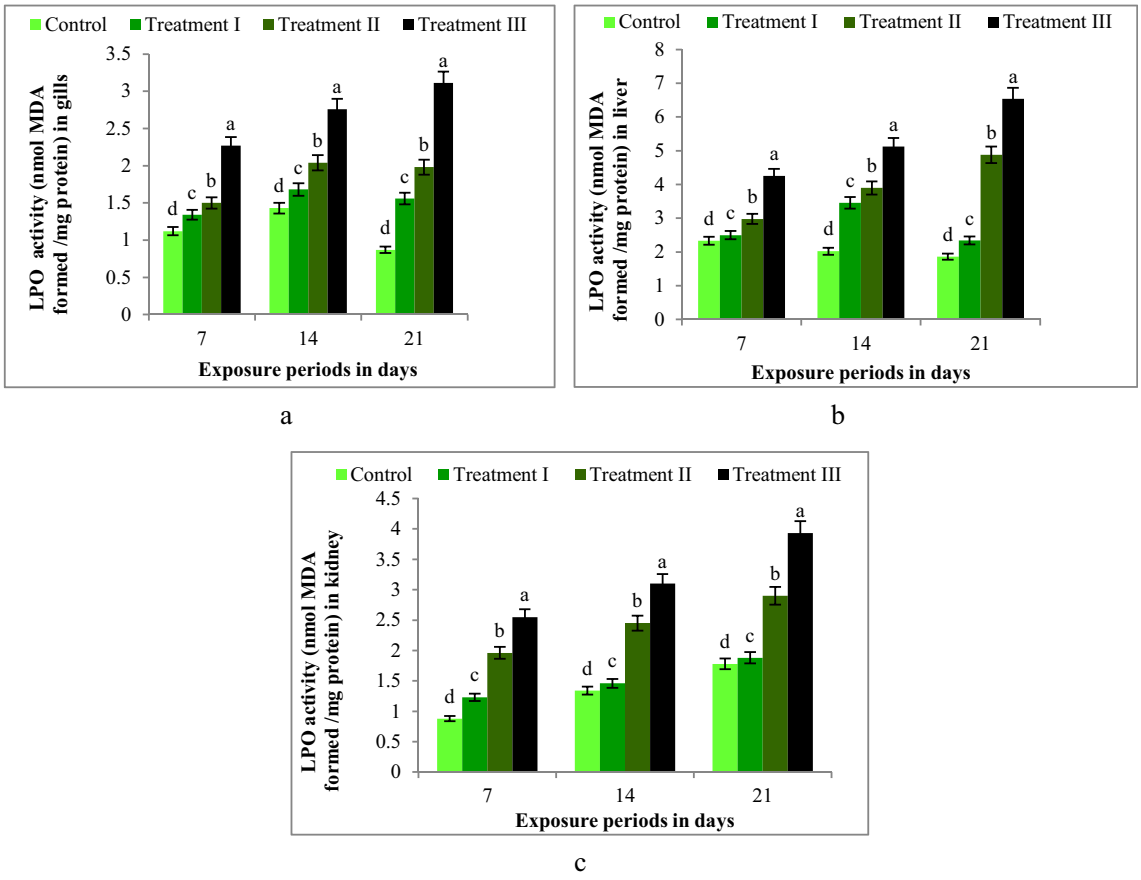

Fig. 7 LPO activity in the gills (**a**), liver (**b**), and kidney (**c**) tissues of C*irrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

such as *Silurus glanis* and Atlantic salmon (Jakimska et al. 2013; Arukwe et al. 2018). The alteration of electrolytes in living organisms might be due to dehydration and damage in liver or kidney tissues (Curran et al. 2017).

Structural changes in the tissues are widely used as an ideal biomarker in toxicology. Adverse changes that occurred in biochemical and physiological parameters could cause histological alterations in tissues of fish (Arellano et al. 2001; Fanta et al. 2003). Most of lipophilic chemical substances tend to cross the cell membrane actively and accumulate in the cell. This could affect the amino acid, fatty acid, and lipid metabolism, which are known to be the major components in energy regulation for normal homeostasis and function of cells.

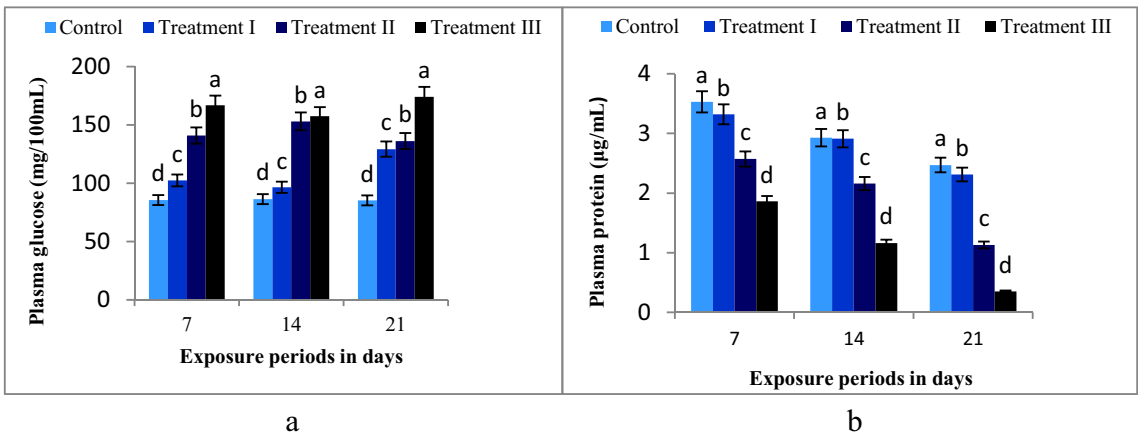

Fig. 8 Plasma glucose (**a**) and protein (**b**) level of C*irrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

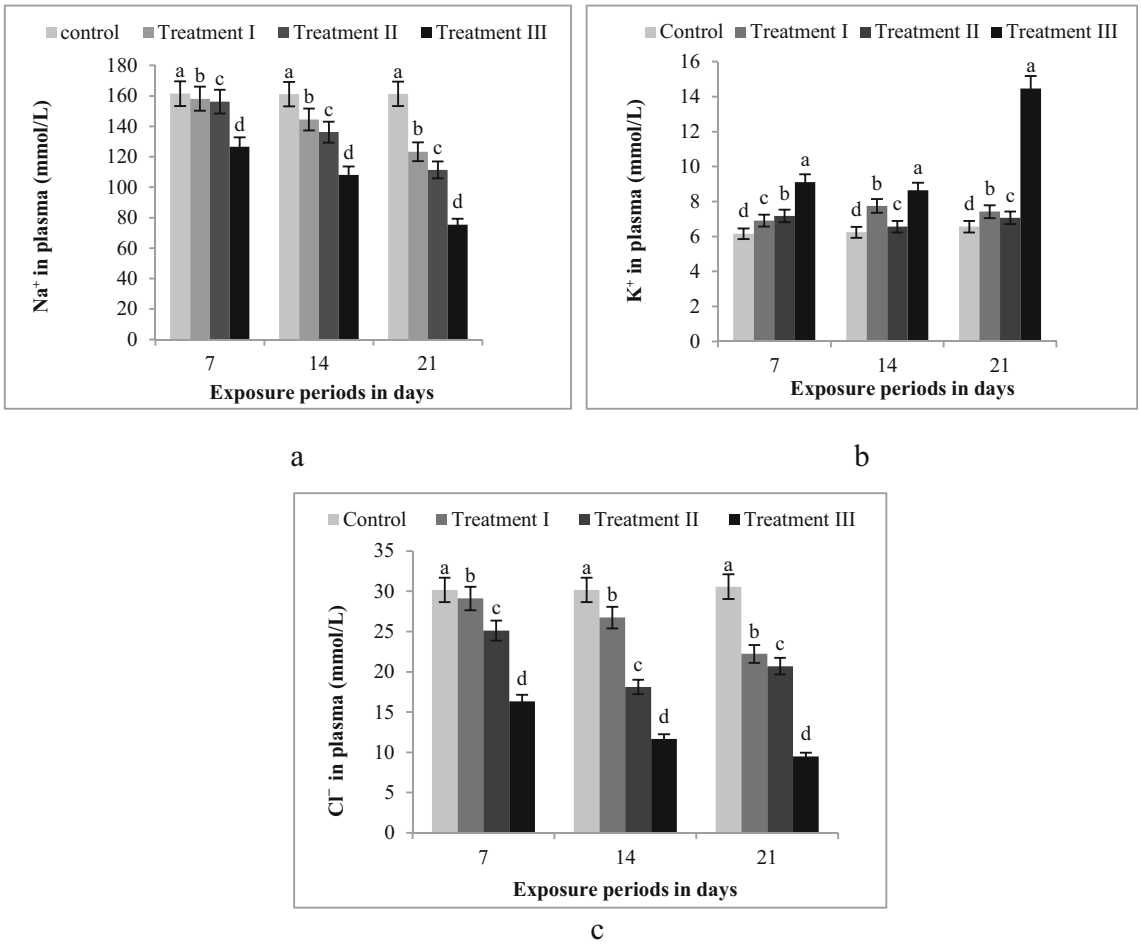

Fig. 9 Plasma Na^+(**a**), K^+ (**b**), and Cl^- (**c**) level of *Cirrhinus mrigala* under long-term exposure periods. TCEP treatment I (0.04 mg/L), treatment II (0.2 mg/L), and treatment III (1 mg/L). Different letters on the bars indicate significant difference at $p < 0.05$

Gill is the primary organ in contact with waterborne contaminants. It also plays a vital role in osmoregulation (Marigoudar et al. 2018). A series of structural anomalies such as epithelial lifting (EL), hyperplasia (H), mucus accumulation (M), and vacuolation (V) were observed in the gills of TCEP-treated fish. Since TCEP is a lipophilic substance, it could cross the epithelial membrane and altered the biochemical component in the layer and that could be the reason for occurring of epithelial lifting and degeneration of cells in primary lamellae. Fusion of cells could generate more free space; thus, vacuolation occurred in TCEP-treated fish. In aquatic animals, OPFRs usually absorbed from the water by gill and tend to accumulate in gill (Saeger et al. 1979; Hou et al. 2016).

Liver is a second target organ for most of the compounds, where the biotransformation and detoxification take place. In the present study, TCEP included increased in fat deposits, pyknotic nuclei, vacuolization, necrosis, enlarged sinusoidal vessels, and congestion. Similarly, structural changes in the liver tissues of *Danio rerio* exposed to triphenyl phosphate (Du et al. 2016). Tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) concentration caused structural alterations in liver such as vacuolization and apoptosis in zebrafish (Liu et al. 2016). The ability of liver to degrade various toxicants results in subsequent structural damage of liver tissues (Bruslé and Anadon 1996; Tabassum et al. 2016). And also, kidney is a major excretory organ where protein reabsorption occurs, where the compounds either parent or metabolites reach the kidney. In the present study, TCEP induced changes in kidney such as hypertrophy in the epithelial cells of renal tubes (HER), shrinkage of glomeruli (SG), expansion of Bowman's capsule (EBC), and degenerated tubular epithelium (TD). TCEP at high concentration (10 mg/kg) damage the structure and function of the intestinal epidermal cells, which may lead to multiple biochemical changes in earthworm (Yang et al. 2018a). Several structural anomalies were noted in *Xenopus tropicalis* embryos upon exposure to FRs (Zhang et al. 2016). Changes in LPO activity observed in this study reveal the action of free radicals on

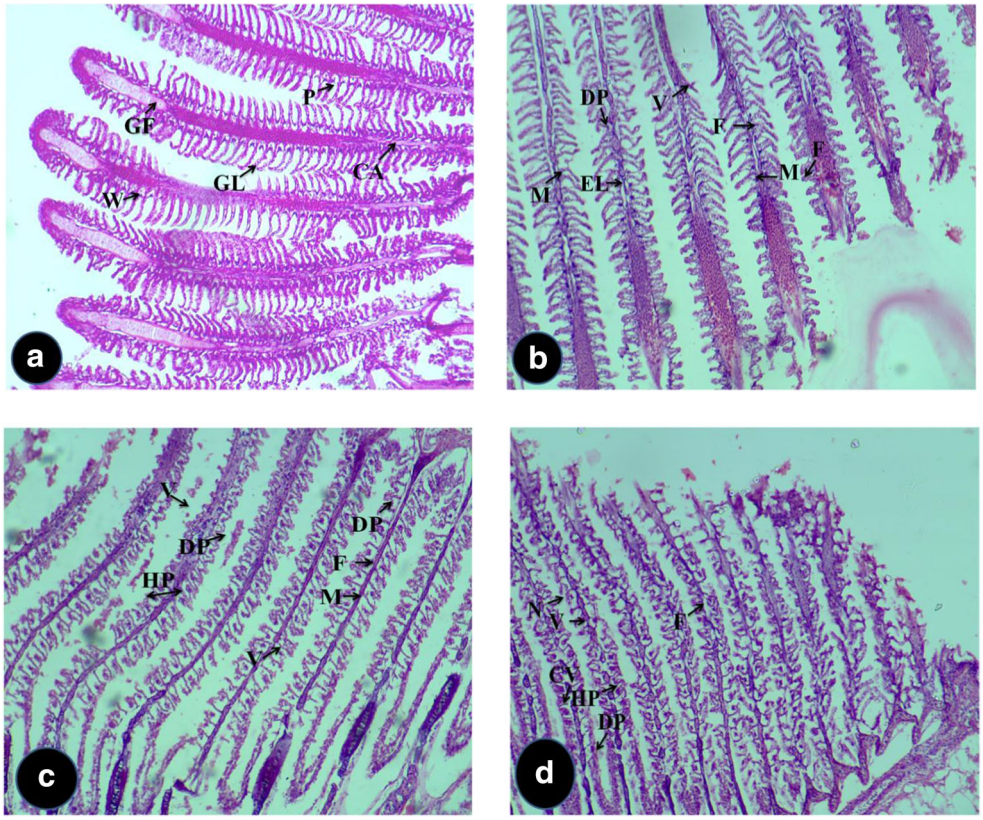

Fig. 10 Histological sections of gills of *C. mrigala*. **a**) control group, with a normal gill filament (GF), gill lamella (GL), cartilage (CA), pillar cells (P), erythrocytes (W). A series of morphological anomalies (epithelial lifting (EL), hyperplasia (H), degeneration of cells in primary lamellae (DP), fusion (F), mucous accumulation (M), Vacuolation (V)) were noticed in TCEP treatment (treatment I (**b**), treatment II (**c**), and treatment III (**d**)) groups. (HE × 40)

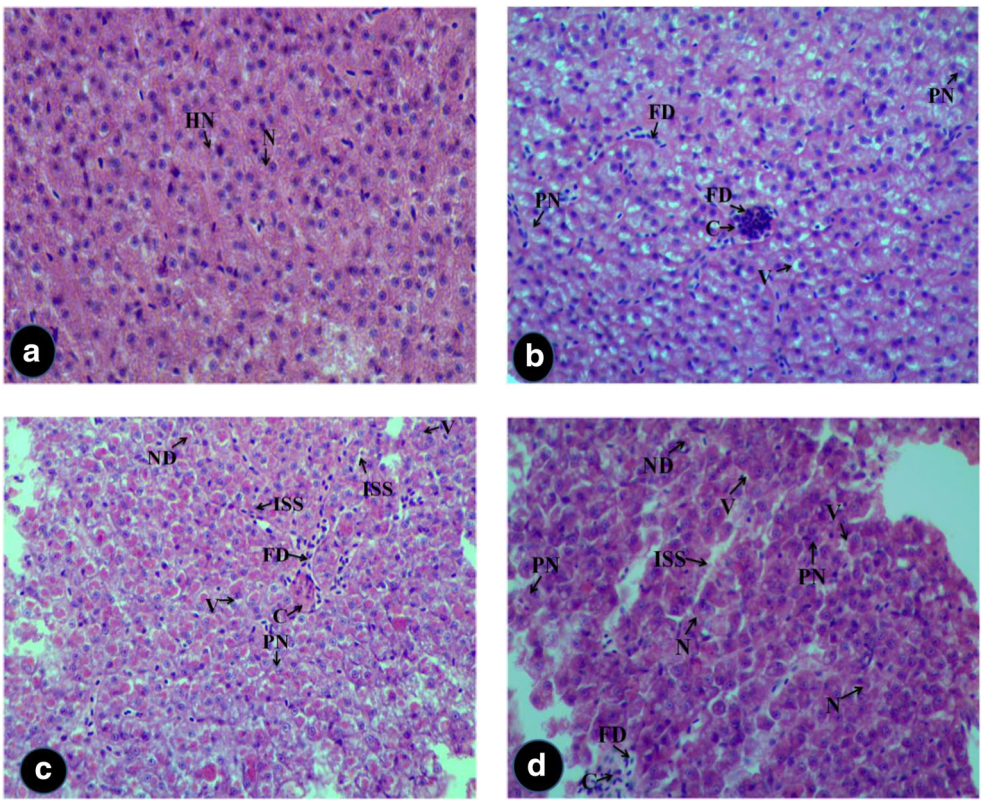

Fig. 11 Histological sections of liver of *C. mrigala*. **a**) control group, with a normal hepatocyte (HN), and nucleus (N). A series of morphological anomalies (pyknotic nuclei (PN), fat deposition (FD), necrosis (N), increased sinusoids vessels (ISS), congestion (C) and vacuolation (V)) were noticed in TCEP treatment (treatment I (**b**), treatment II (**c**), and treatment III (**d**)) groups. (HE × 40)

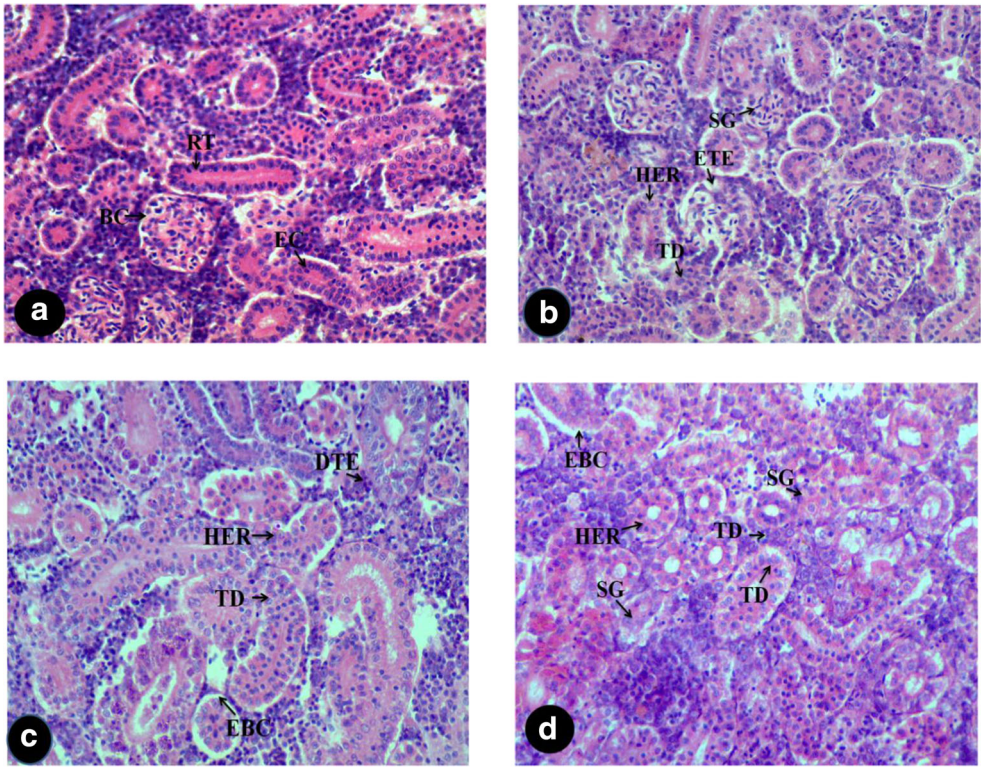

Fig. 12 Histological sections of kidney of *C. mrigala*. **a**) control group, with a normal Bowman's capsule (BC), renal tubule (RT), and glomerulus (EC). A series of morphological anomalies (hypertrophied epithelial cells of renal tubes (HER), shrinkage of glomeruli (SG), expansion of Bowman's capsule (EBC), degenerated tubular epithelium (TD)) were noticed in TCEP treatment (treatment I (**b**), treatment II (**c**), and treatment III (**d**)) groups. (HE × 40)

Table 1 Morphological analysis of gill tissues of *C. mrigala* exposed to different concentrations (0.04, 0.2, and 1 mg/L) of TCEP. The morphological anomalies were represented in symbol based on their severity

Organ	Pathology	Control	Treatment I	Treatment II	Treatment III
Gills	Epithelial lifting	−	+	++	+++
	Hyperplasia	−	+	++	+++
	Degeneration of cells in primary lamellae	−	+	++	+++
	Lamellar fusion	−	+	++	+++
	Mucus accumulation	−	+	++	+++
	Vacuolation	−	+	++	+++

(−) no anomalies examined, (+) anomalies examined

the lipid membrane of the cell, which might result in a series of histological anomalies in TCEP-treated fish. Thus, antioxidants, hormonal, and biochemical alterations observed in the present study might cause some morphological changes in the tissues of TCEP-treated fish.

Table 2 Morphological analysis of liver tissues of *C. mrigala* exposed to different concentrations (0.04, 0.2, and 1 mg/L) of TCEP. The morphological anomalies were represented in symbol based on their severity

Organ	Pathology	Control	Treatment I	Treatment II	Treatment III
Liver	Pyknotic nuclei	−	+	++	+++
	Fat deposition	−	+	++	+++
	Necrosis	−	+	++	+++
	Increased sinusoids	−	+	++	+++
	Vessels				
	Congestion	−	+	++	+++
	Vacuolation	−	+	++	+++

(−) no anomalies examined, (+) anomalies examined

Table 3 Morphological analysis of kidney tissues of *C. mrigala* exposed to different concentrations (0.04, 0.2, and 1 mg/L) of TCEP. The morphological anomalies were represented in symbol based on their severity

Organ	Pathology	Control	Treatment I	Treatment II	Treatment III
Kidney	Hypertrophied epithelial cells of renal tubules	−	+	++	+++
	Shrinkage of glomeruli	−	+	++	+++
	Expansion of Bowman's capsule	−	+	++	+++
	Tubular degeneration	−	+	++	+++

(−) no anomalies examined, (+) anomalies examined

Conclusion

The findings of the present investigation indicate that the TCEP at tested concentrations induced endocrine disruption especially in the thyroid hormone levels and caused oxidative stress in fish. Furthermore, the structural damage mediated by TCEP resulted in high energy demand that ultimately increased the energy metabolism in fish. The alterations of these parameters can be effectively used to monitor the impact of FRs on aquatic environment. The data of the present study could be used as a toxicity data for other organophosphorus flame retardants on fishes. From the result, we conclude that the levels of FRs in aquatic environment should be monitored.

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