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Antimicrobial agents, triclosan, chloroxylenol, methylisothiazolinone and borax, used in cleaning had genotoxic and histopathologic effects on rainbow trout

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HIGHLIGHTS

- MIT, TRC and PCMX were genotoxic.
- Genes were significantly regulated by TRC, PCMX, MIT and BRX.
- TRC, PCMX, MIT and BRX caused histopathological effects.
- Chronic toxic effect of TRC, PCMX, MIT, and BRX is high in rainbow trout.

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ABSTRACT

Triclosan (TRC), chloroxylenol (PCMX) and methylisothiazolinone (MIT) have been commonly used as an antimicrobial in soaps while borax (BRX) is used in household cleaning. After using these chemicals, they are washed down drains and getting into the aquatic ecosystem in which they may affect aquatic living organisms. In the present study, the chronic effects of TRC, PCMX, MIT and BRX on genotoxicity, gene expression and histopathology of rainbow trout (*Oncorhynchus mykiss*) were evaluated for 40 days under semi static condition. The comet assay results indicated that MIT, TRC and PCMX caused significant DNA damage to erythrocytes of the fish. Transcription of *SOD*, *GPX*1, *GPX*2, *GST*A, *HSP90*BB, *HSP90*BA, *CAT*, and *HSC70*A genes were significantly regulated as a result of TRC, PCMX, MIT, and BRX exposure except PCMX exposed *GSTA* gene. Histological lesions were detected in gills, spleen liver, and trunk kidney of the fish. Lamellar fusion, hyperplasia and epithelial necrosis in gills, melanomacrophage centers and splenic necrosis in spleen, pyknotic nucleus, fat vacuoles, necrotic hepatocytes in liver, cloudy swelling in the tubules, renal tubule epithelial cells degeneration, glomerular capillaries dilation and glomerulus degeneration in kidney, were observed. Our study demonstrates the chronic toxic effect of TRC, PCMX, MIT, and BRX is high in rainbow trout. Therefore, we should be more careful when using these chemicals for cleaning in order to protect aquatic environment.

1. Introduction

Antibacterial chemicals also known as antimicrobial pesticides are produced for disinfection, sanitization, decreasing or mitigation of growth or protection of inert objects caused by microorganisms. Nowadays, different kinds of active ingredients have been used in antimicrobial products and they are marketed in many types of formulations including: liquids, concentrated powders, sprays and gases (EPA, 2016). Triclosan, borax and chloroxylenol are commonly used for antibacterial and deodorant effect in consumer products. These chemicals used in hand soaps have been washed down drains and getting into the water system and affecting aquatic environment. These chemicals resistant to degradation and it has been found in streams and wastewater all around the world (Bedoux et al., 2012; Carey and McNamara, 2015; Saleh et al., 2011). There are also some concerns that they have potential to spread antibiotic resistance (Carey and McNamara, 2015; Saleh et al., 2011).

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The majority of liquid hand soaps and bars use triclosan (TRC) as an active ingredient (Perencevich et al., 2001). Triclosan is a phenoxyphenol antimicrobial, it also has some antiviral and antifungal activity (Bhargava and Leonard, 1996). Recently, it has been placing the chemical all sorts of home items such as in wipes, hand gels, cutting boards and mattress pads to eradicate any bacteria. Increased household sewage discharges can impact water quality in the near shore, an area that is essential for the survival of healthy aquatic organisms (Halden, 2014). TRC is determined in water, wastewater, soil, and sediments (Bedoux et al., 2012; Mavri et al., 2012). This chemical is lipophilic, persistent environmental pollutant thus poses a high risk of bioaccumulation. It is toxic to living organisms in aquatic environments, particularly immediately downstream of effluents from household wastewaters (Brausch and Rand, 2011). Triclosan was detected in many fish species and surface water (Benotti et al., 2009; Dougherty et al., 2010). It has been demonstrated laboratory evidence of triclosan resistance with antibiotics among multiple species of bacteria (Aiello et al., 2004, 2005). However, there are no adequate studies about whether occurrence of antibiotic resistance in environment is associated with the growing use of soaps containing triclosan.

Boric acid (BRX) is a naturally occurring chemicals and found in low concentrations in the environment (EPA, 1998). It is a crucial nutrient for many living organisms although it has become an environmental contaminant due to its widespread use (Topal et al., 2016). Borax has been used in many detergents, cosmetics, and enamel glazes. The aquatic organisms like fish are very sensitive to borax exposure (Schoderboeck et al., 2011). It is toxic to aquatic organisms and toxic concentration varies over a wide range depending on the species and the test conditions (Loewengart, 2001; Schoderboeck et al., 2011). Toxicity of borax was manifested by generalized focal seizure disorders, irritability, and gastrointestinal disturbances. The most common findings were edema and congestion of the brain and meninges. Other common findings included liver enlargement, vascular congestion, fatty changes, swelling, and granular degeneration (Linden et al., 1986; Litovitz et al., 1988; Penland, 1994).

Chloroxylenol (PCMX) and methylisothiazolinone (MIT) are other antimicrobial chemicals using in different products. PCMX is in use as an ingredient to control microorganisms, algae and fungi in paints, emulsions, washing tanks and adhesives. It is also commonly used in antibacterial soaps, wound-cleansing applications and household antiseptics (Bruch, 1986). Toxic effects of PCMX are ranging from low to high, depending on the species and test conditions. While freshwater fish are very susceptible to PCMX, aquatic invertebrates are moderately vulnerable (EPA, 1994). Chloroxylenol is a skin and eye irritant. It can changes blood cellular composition after repeating oral and dermal exposure (Guess and Bruch, 1986). The toxicological data for chloroxylenol lacks of genotoxicity and carcinogenicity (Yost et al., 2016). MIT is a widely used industrial and household biocide and preservative in personal care and cosmetics products such as hand and body lotions, moisturizers, sun tanning lotions and some rinse-off products. In water systems MIT is commonly used antimicrobial to control fungi, slime-forming bacteria and algae. MIT is allergenic and cytotoxic chemical. Chronic exposure to MIT and related compounds may damage to the emerging nervous system (Du et al., 2002). It may cause DNA damage, and affect the enzyme activity as well. Some of industrial products including MIT has been found in estuarine and coastal environments (Voulvoulis et al., 2002).

Because of the widespread use of antimicrobial chemicals, the cellular and molecular effects of these pollutants on fish need to be evaluated. Despite many studies focused on the effects of these chemicals on human is sufficient (Levy, 2002; Pirard et al., 2012), the effects on aquatic ecosystems are not fully illuminated. Although the antimicrobial properties of the disinfectants against some pathogenic microorganisms, ecological risk of this chemical when mixed to receiving water should be thoroughly examined. The objectives of this study were to investigate (1) the chronic toxic effects, (2) histopathological effects (on liver, kidney and gill tissue), (3) blood DNA damage and (4) gene expression effects of TRC, PCMX, MIT, and BRX on rainbow trout (*Oncorhynchus mykiss*).

2. Materials and methods

2.1. Test compounds

Triclosan ($C_{12}H_7Cl_3O_2$, Sigma-Aldrich), chloroxylenol (4-Chloro-3,5-dimethylphenol, Merck Millipore), methyloisothiazolinone (2-Methyl-4-isothiazolin-3-one, Sigma-Aldrich) and borax ($Na_2B_4O_7$, Sigma-Aldrich) were commercially obtained and selected as antibacterial agents for chronic toxicity tests.

Stock solutions of TRC and PCMX were prepared by diluting with ethanol (Merck, ACS reagent, absolute) while MIT and BRX were diluted with distilled water to obtain desired concentrations. During the experiment, daily prepared fresh stock solutions were used.

2.2. Fish

All experiments described in this study were approved by the Institutional Animal Care and Use Committee at Karadeniz Technical University. Rainbow trout (17.77 ± 3.92; 12.04 ± 0.90 cm; Mean ± SD), obtained from Karadeniz Technical University, Faculty of Marine Sciences, were acclimated to laboratory conditions in recirculating systems (200 L) prior to experiments for 30 days. During acclimation and chronic toxicity tests, fish were held under a photoperiod (12 h of light and 12 h of darkness) and fed with commercial trout pellets (4% BW) daily.

During chronic exposure to the antimicrobial chemicals, water characteristics such as temperature, pH, dissolved oxygen, nitrite and ammonia (Boyd and Tucker, 1992) were measured daily in each treatment (Table 1).

2.3. Experimental design

Prior to this study, 96 h LC_{50} concentration for TRC, PCMX, MIT and BRX were found 0.05 mg L^{-1}, 0.043 mg L^{-1}, 0.072 mg L^{-1} and 1 g L^{-1}, respectively (unpublished data). One-hundredth of LC_{50} concentrations of antimicrobials were selected for chronic exposure. Thus, 0.48 ± 0.2 μg L^{-1} of TRC, 4.2 ± 0.9 μg L^{-1} of PCMX, 6.8 ± 1.1 μg L^{-1} of MIT and 8.9 ± 1.8 mg L^{-1} of BRX actual concentrations were used for chronic exposure. MIT and BRX were dissolved in 50 mL distilled water while TRC and PCMX were dissolved in 50 mL ethanol then added test water. Fish were stocked into 3 × 40-l aquaria at a rate of 20 fish/aquarium. Fish were subjected to chronic exposure of TRC, PCMX, MIT and BRX for 40 days after acclimation period. While one of two control groups have only the test water, the other control group was created for TRC and PCMX experiments and prepared with the same amount of ethanol that added to prepare chemical solutions in test groups. During the chronic exposure, fifty-six percent of test solutions and water of control groups were renewed daily (Boran et al., 2010). Fish were sacrificed at 40th day for experimental analysis.

At the beginning of the experiment and then every two days, water samples were taken to determine the actual concentrations of the chemicals in water. HPLC was used for determination of the concentrations of TRC (Zhao et al., 2011), PCMX (Gatti et al., 1997), MIT (Baranowska and Wojciechowska, 2013) and BRX (EPA, 2013) in water.

Table 1
Water quality parameters (mean ± SD) during the study.

Water characteristics	Water[a]	Control	Control[b]	BRX	MIT	TRC	PCMX
Temperature (°C)	15.1 ± 0.2	15.1 ± 0.1	15.1 ± 0.1	15.1 ± 0.2	15.1 ± 0.2	15.1 ± 0.1	15.1 ± 0.2
pH	8.14 ± 0.21	7.90 ± 0.18	7.82 ± 0.34	8.43 ± 0.11	8.23 ± 0.27	7.92 ± 0.30	7.85 ± 0.25
Dissolved oxygen (mg/L)	9.4 ± 0.3	9.0 ± 0.2	9.1 ± 0.2	8.9 ± 0.1	8.7 ± 0.3	8.8 ± 0.2	8.4 ± 0.3
Total ammonia (ng/L)	1.0 ± 0.02	21.8 ± 3	22.1 ± 0.9	24.5 ± 5.7	12.7 ± 2.1	11.6 ± 1.2	21.3 ± 3.2
Nitrite (ng/L)	5.0 ± 1.7	30 ± 1.8	8.4 ± 3.3	27 ± 8.9	26 ± 6.5	23 ± 2.3	24 ± 9.1

[a] Untreated water.
[b] Ethanol containing control.

2.4. Histopathology

After 40 days of chronic exposure to TRC, PCMX, MIT and BRX, small part of gills, kidney and liver were removed from fish and immediately fixed in neutral buffered formalin solution (10%) for 48 h. Organs were rinsed with alcohol series and cleared in xylene. After paraffin sections, a thickness of 5 μm were stained with hematoxylin and eosin (Luna, 1968). Histopathological lesions were examined with a light microscope. The scores were reported as follows: none: − (0 lesion), mild: + (1–4 lesions), moderate: ++ (5–8 lesions) and severe: +++ (≥9 lesions) (Boran et al., 2012).

2.5. Comet assay

Comet assay was conducted according to the method used by Altinok et al. (2012). At the end of the chronic exposure, 0.5 mL of blood was taken from caudal vein of 10 fish for each experiment and control group. To obtain 100 cells/ml blood was diluted with phosphate buffered saline (PBS) and 10 μL cell suspension was mixed with 75 μL of 0.5% low melting point agarose (LMPA). Each slide was coated with 80 μL cell/LMPA suspension and covered with coverslip then harden for 10 min at 4 °C. Slides were coated again with 80 μL of 0.5% LMPA and harden. Blood of control fish were used as a positive control. After positive control and control slides (negative) formed as above, only positive control slide was exposed to hydrogen peroxide for 5 min. After slides were immersed in lysis solution at 4 °C, for 1 h in the dark, slides were placed in electrophorese box with alkaline electrophoresis buffer (pH > 13) at 4 °C. All the slides were exposed to 24 V (0.74V cm^{-1}, 300 mA) for 30 min in the dark. After this step, slides were stained with ethidium bromide (1X) for 10 min and washed with distilled water. Fluorescent microscope was used to observe DNA damage. Comet Software (v1.3, OpenComet) was used for comet scoring. Tail DNA and Cell DNA Intensity were taken from 60 to 80 cells per fish. Tail moment was used to measure DNA damage as following

$$\text{Tail DNA\%} = 100 \times \text{Tail DNA Intensity/Cell DNA Intensity}$$

2.6. Gene expression

2.6.1. RNA isolation

Before chronic exposure (time zero control) and after chronic exposure, 0.5 g of liver and kidney were taken from 8 fish per treatment and stored −80 °C prior to isolation. Trizol method (Thermo Fisher) was used to isolate total RNA. Total of 60 mg tissue taken from the liver (30 mg) and kidney (30 mg) were put in 1 mL Trizol solution and 4 small beads containing Eppendorf tube. Samples were homogenized with bullet blender for 4 min. Then 250 μl chloroform was added and shacked vigorously for 15 s, centrifuged at 14000 × g for 5 min. The aqueous phase was carefully removed and RNA precipitate was obtained after isopropanol addition. The RNA absorbance was measured at 260 nm after DNase treatment. DNase treated RNA can then be used immediately for the reverse transcription reaction. The integrity of RNA samples was verified by 1% agarose gel.

2.6.2. Reverse transcription

The High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) was used with random primer for initiating cDNA synthesis of DNase treated RNA. Each sample of RNA (1 μg) was run with reverse transcriptase. After prepare the 2× reverse transcription master mix, RNA was added to the 2× RT master mix to create a 1× mix. Samples were incubated at 25 °C for 10 min, 37 °C for 120 min and then 85 °C for 15 min, respectively. After fully inactivating remaining enzymes, the reverse transcription reactions (cDNA) were directly used for RT- PCR or stored at −20 °C for long term storage.

2.6.3. Primers

After exposing rainbow trout to TRC, PCMX, MIT and BRX, the following gene were studied. Cu/Zn-superoxide dismutase (*SOD*), glutathione peroxidase 1 (*GPX*1), glutathione peroxidase type 2 (*GPX*2), glutathione S-transferase A (*GST*A), heat shock 90 kDa protein 1 beta isoform b (*HSP90*BB), heat shock 90 kDa protein 1 beta isoform a (*HSP90*BA), heat shock 70 kDa protein 8 isoform a (*HSC70*A) and catalase (*CAT*). genes from. Primers were designed (Table 2) from these genes by using the Primer-BLAST program available at NCBI.

2.6.4. Quantitative real-time PCR

qRT-PCR analyses was performed in Exicycler 96 (Real-Time Quantitative Thermal Block, Korea). The primer sequences used for the amplification of the target genes showing the change in enzyme activity were presented in Table 2. Primers were checked for their characteristics such as hairpins, self-annealing, complementarity and secondary structure with OligoCalc (Northwestern University, USA). Samples were prepared duplicate in qRT-PCR for each sample. Each PCR reaction mix (25 μl total) included 12.5 μL of Hot Start Taq 2X Master Mix (NEB, BioLab), 1 μl cDNA, 10 pmol of each primer, 1 μL of 20X EvaGreen (Biotium, Hayward, CA, USA) and 9.5 μl of dH_2O. After pre-incubated at 95 °C for 1 min, samples were incubated at 95 °C for 20 s, 58 °C for 20 s, 68 °C for 45 s, respectively and after scan, this cycle was repeated 35 times. At the end of the reaction, melting curve analysis was done to control for the specificity of the reaction. In each reaction plate, negative control reactions without any template was run to check contamination and dilution series were conducted to determine efficiency of the target and reference genes primer pairs. qPCR products were also run on the 1.5% agarose gel to check fragment size. Standard curves were created for target genes and reference gene (Elongation factor 1 alpha (*EF*1) gene and beta actin gene) with tenfold serial dilutions of cDNA. Each sample was run in triplicate and average value was used to calculate $\Delta\Delta C_T$.

EF1 alpha gene was used as a reference gene for normalization of target gene transcripts. Amplification efficiencies of the target and reference genes were similar; therefore, method developed by

Table 2
Specific primer pairs for Cu/Zn-superoxide dismutase (*SOD*), glutathione peroxidase 1 (*GPX*1), glutathione peroxidase type 2 (*GPX*2), Glutathione S-transferase A (*GSTA*), Heat shock 90 kDa protein 1 beta isoform b (*HSP90*BB), Heat shock 90 kDa protein 1 beta isoform a (*HSP90*BA), heat shock 70 kDa protein 8 isoform a (*HSC70*A), catalase (*CAT*) and Elongation factor 1 alpha (*EF*1) genes from rainbow trout.

Gene	Accession No	Primer sequence (5' –> 3')	Product size (bp)	Annealing temp. (°C)
SOD	AF469663.1	F-GTTGACTCTCACTGGACCCG R- TCCTCGTTGCCTCCTTTTCC	90	59.96
*GPX*1	NM001124525.1	F-TGGAGAGCCATTCAAGCGTT R- CTCCCCACCATTGCTTCTGT	126	59.96
*GPX*2	AY622862.1	F-TGGAGAGCCATTCAAGCGTT R- CTCCCCACCATTGCTTCTGT	126	59.96
GSTA	NM001160559.1	F-GAGGTGGGTTCTTACCTGGC R- TAGACAGCCCAAAGCGGAAG	94	60.04
*HSP90*BB	AB196458.1	F-TGAAGAAATGCGCCAAGAGGA R- TCCGTCAGACTTTCGTAGCG	174	59.83
*HSP90*BA	AB196457.1	F-AGCCTCACGTTTTCCAATCG R- AGATGCGTTGCCCACCATTA	157	60.03
*HSC70*A	AB196460.1	F-TGTCTAAGGGACCAGCAGTC R- CGTAGCTTGGAGTGGTCCTG	123	60.11
CAT	FJ226382.1	F-CAAGGCCAAGGTGTTTGAGC R- GTAAGGTCCCAGTTGCCCTC	153	59.97
*EF*1 Alpha	AF498320.1	F-ATGCCCCTGTACTGGATTGC R- TGGGGGCATCCTCAAGTTTC	105	60.01

Livak and Schmittgen (2001) was used to calculate relative gene expression.

C_T of the gene of interest to that of the reference gene (*EF*1) were normalized as

$$\Delta C_{T(\text{treated sample})} = C_{T\ (\text{gene of interest in treated sample})} - C_{T(EF1\ \text{in treated sample})}$$

$$\Delta C_{T(\text{untreated control})} = C_{T(\text{gene of interest in untreated control})} - C_{T(EF1\ \text{in untreated control})}$$

$$\Delta\Delta C_T = \Delta C_{T(\text{treated sample})} - \Delta C_{T(\text{untreated control})}$$

Normalized expression ratio was calculated as $2^{-\Delta\Delta C_T}$

2.7. Statistical analysis

Experimental data were given as means ± the standard deviations (SD) for each experiment. IBM SPSS V22.0 (SPSS Inc., Chicago, IL, USA) software was used for statistical analyses. For bioavailability data, Kolmogorov-Smirnov's and Bartlett's tests were used for normality and homogeneity of variance, respectively. One-way general linear model analysis of variance and a post-hoc Tukey's test were used to analyze fold change and multiple comparisons, respectively. $P \leq 0.05$ was considered as significant level.

3. Result

During the experiment water quality were measured daily. After fish exposed to TRC, PCMX, MIT, and BRX for 40 days, eight fish were random sampled for experimental analysis. Blood, liver, kidney for genetic damage and liver, kidney, and gill were used for tissue disorder. No abnormal behavior was observed on fish except low feeding rate during the exposure.

3.1. Histopathological examination

After chronic exposure to TRC, PCMX, MIT, and BRX, gill, spleen, liver, and kidney tissue were dissected immediately for histopathological examination (Table 3). Histopathologically, some lesions were observed in control groups containing alcohol while no changes were seen in the normal control. Lamellar fusion, hyperplasia and epithelial necrosis were observed in fish gills (Fig. 1). Melanomacrophage centers and splenic necrosis in spleen sections (Fig. 2) and pyknotic nucleus, degradation in hepatic cell nuclei, fat vacuoles and necrotic hepatocytes were observed in liver to varying degrees (Fig. 3). Kidney showed the most symptoms compared to gill, spleen and liver. In kidney tissue, cloudy swelling in the tubules, renal tubule epithelial cells degeneration, glomerular capillaries dilation, pycnotic nuclei in the hematopoietic tissue and glomerulus degeneration were intensively detected symptoms (Fig. 4). All of these symptoms detected in the experimental groups were observed in the control group containing alcohol as well as in the low severity.

3.2. Comet assay

After examining 1500 red blood cells for the presence of comet (comets tail intensity tail moment, tail migration and tail length) from each exposure group, TRC, PCMX, MIT, and BRX cause significant DNA damage to red blood cells of the fish. DNA damage (tail DNA %) for the negative control (water) and negative control (water + alcohol) were 39.72 and 127.01, respectively (Table 4) while the positive control was 313.56. MIT was the most induced DNA damage in red blood cells of fish when compared with other chemicals. DNA damage scores for MIT was 544.45. TRC and PCMX (dissolved with alcohol) had similar genotoxic activity on blood DNA and their damages were 397.83 and 421.88, respectively. BRX posed less DNA damage than others with 254.81. The comet assay results indicated especially MIT, TRC and PCMX have mutagenic effects on blood DNA of fish although its concentrations used in too low values for chronic exposure.

3.3. Gene expression

Efficiency of each reaction was ranged between 97% and 102%. No unspecific or more then one fragment was observed after running qPCR products on agarose gel. The fold change in expression of the SOD1, GPX1, GPX2, GSTA, HSP90BB, HSP90BA, and HSC70A genes were determined after normalizing with the reference gene and control groups (Table 5). After normalization, transcription of SOD, GPX1, GPX2, GSTA, HSP90BB, HSP90BA, CAT, and HSC70A genes were significantly regulated as a result of TRC, PCMX, MIT, and BRX exposure except PCMX exposed GSTA gene. After exposing fish to PCMX, SOD, GPX2, HSP90BB and HSC70A genes

Table 3
Histopathological findings of rainbow trout after exposing to antibacterial agents for 40 d. Lesions were scored based on their severity (−: none,+: mild, ++: moderate, +++: severe).

Histopathological effects	Control	Control (Alcohol)	BRX	PCMX	MIT	TRC
	Gill					
Lamellar fusion	−	+	++	−	+	+
Hyperplasia	−	+	+++	+	+	+
Epithelial necrosis	−	+	+	+	++	+++
Hypertrophy	−	−	+	−	+	+
Epithelial lifting	−	−	−	+	−	+
Edema	−	−	−	+	−	−
	Spleen					
Melanomacrophage centers	−	+++	+++	+++	+++	+++
Splenic necrosis	−	−	++	−	−	−
	Liver					
Pyknotic nucleus	−	−	++	−	+	−
Degradation in hepatic cell nuclei	−	−	−	++	−	−
Fat vacuoles	−	−	−	+++	+++	−
Necrotic hepatocytes	−	−	−	−	−	+++
	kidney					
Melanin and melanomacrophage centers	−	+	++	+	++	+++
Cloudy swelling in the tubules	−	+	+++	+++	+	+++
Renal tubule epithelial cells degeneration	−	+	+++	+++	++	+++
Glomerular capillaries dilation	−	+	++	++	++	+++
Pycnotic nuclei in the hematopoietic tissue	−	+	++	+++	++	+++
Glomerulus degeneration	−	+	++	+++	++	+++

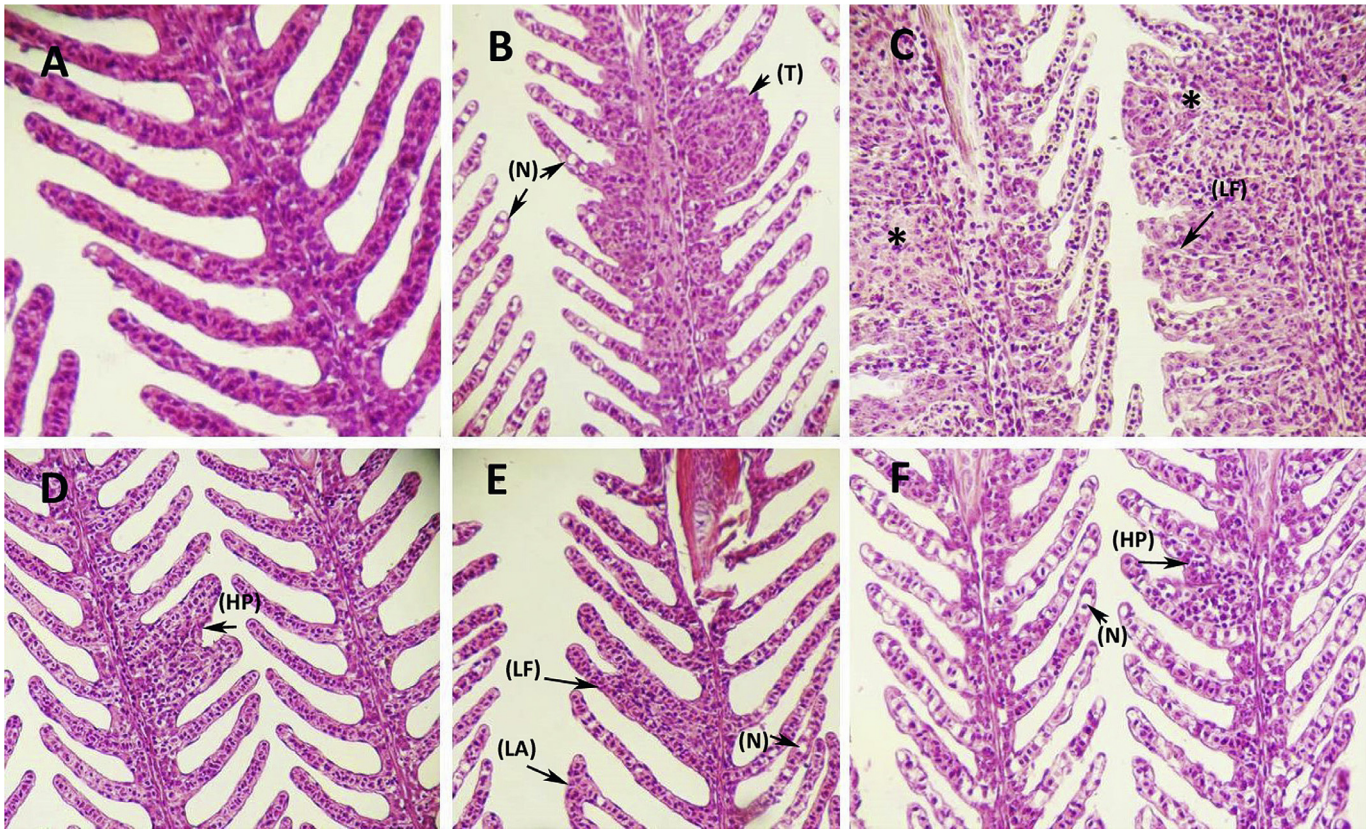

Fig. 1. Histopathology of rainbow trout gills stained with H&E 40 days after exposure to (a) water (control), (b) alcohol, (c) BRX, (d) PMCX, (e) MIT, and (f) TRC. Histopathologic lesions were necrosis (N), thrombosis (T), lamellar fusion (LF), wide hyperplasic area (*), hyperplasia (HP), and lamellar apposition (LA).

were down regulated. Also, BRX, PCMX and TRC exposed fish CAT gene was down regulated while GSTA gene expression was not affected by PCMX (Fig. 5). All the genes except GPX1 and HSP90BA were down regulated after exposing PCMX. The most down regulated gene was PCMX exposed GPX2 gene. The rest of the genes were up regulated and BRX exposed fish HSP90BA gene was the most up regulated by 2129.69-fold. The most affected genes were GPX2 (0.01-fold) and GPX1 (593.79-fold) for PCMX, CAT (0.96-fold) and HSP90BA (2129.69-fold) for BRX, GPX2 (664.21-fold) for MIT and CAT (0.56-fold) and 786.10-fold) for TRC (Table 5).

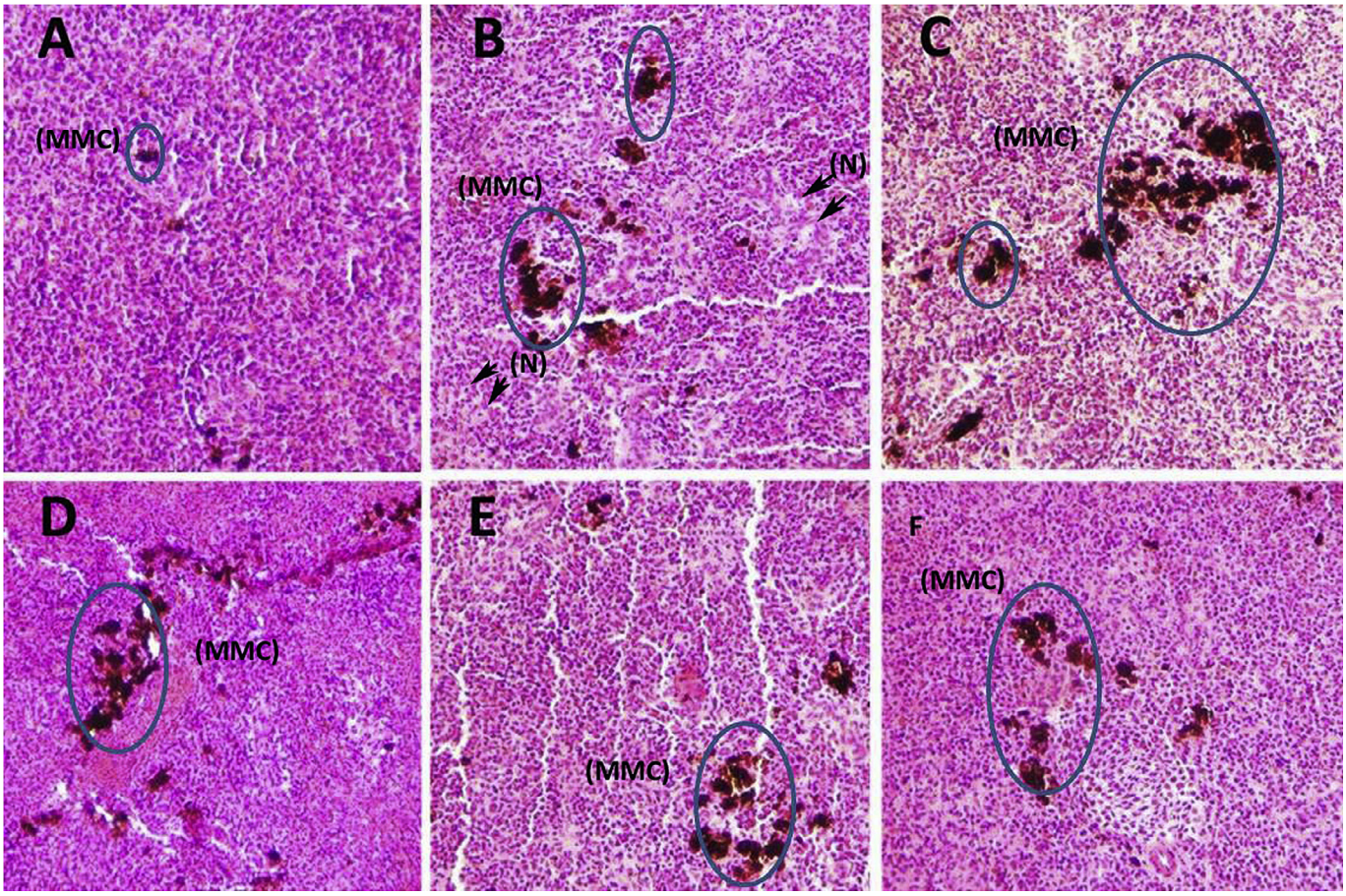

Fig. 2. Histopathology of rainbow trout spleen stained with H&E 40 days after exposure to (a) water (control), (b) alcohol, (c) BRX, (d) PMCX, (e) MIT, and (f) TRC. Histopathologic lesions were necrosis (N) and melanomacrophage centre (MMC).

4. Discussion

Although the use of many protective products had the potential harm to consumer health (Yost et al., 2016), there is not enough data for toxicity or toxic mechanisms of this chemicals. Most of the studies were focused on triclosan and its effects on human health. There is some evidence that triclosan and the other chemicals can disrupt hormone cycles and cause muscle weakness (Halden, 2014). For this reason, U.S. Food and Drug Administration (FDA) banned 19 chemicals including Triclosan used in antibacterial soaps. Many companies have replaced triclosan with one of three other chemicals such as benzethonium chloride, triclocarban or chloroxylenol (PCMX) in their antibacterial products. Triclosan can be found in the water and its concentration has been ranged between 0.0118 μg L^{-1} and 4.3 μg L^{-1} in lakes and wastewater, respectively (MDH, 2014). Triclosan is quite highly toxic to aquatic animals at concentrations below 1 mg L^{-1}, and is particularly toxic to the green algae. Bacteria, fish and crustacean had similar sensitivities towards triclosan toxicity while bacteria and fish were more resistant to triclosan toxicity than that of the microalgae (Tatarazako et al., 2004). Ecotoxicological studies demonstrated that LC_{50} values change between 0.26 and 0.54 mg L^{-1} for fish and EC_{50} values for crustaceans and algae in the range of 0.13–0.39 mg L^{-1} and 1.4–19 μg L^{-1}, respectively (Orvos et al., 2002; Reiss et al., 2002). In the present study, fish were exposed to 0.48 ± 0.2 μg L^{-1} of TRC for 40 days and TRC caused mutagenic effects on red blood cells, had histopathological effects on kidney, liver, spleen and gills, and affected gene expression from 0.56-fold for CAT gene to 786.10-fold for GPX2 gene. Therefore, even low concentration of TRC found in the water can be very dangerous to aquatic organisms.

Chloroxylenol has very low water solubility and volatility (EPA, 1994). However, its persistence in the environment is limited and it can be removed completely from environment in 6 day by biodegradation (ACI, 2014). Both in vitro and in vivo toxicological study demonstrate a lack of genotoxic effects of PCMX which used as an active ingredient in fluids soaps, cosmetics, topical medications, and antiseptics (Yost et al., 2016). Also, shorter-term oral studies do not demonstrate any genic effects of this chemical (EPA, 2009). After chronic dermal exposure, it has no genotoxicity and carcinogenicity (Momma et al., 1988). On the other hand, our results showed that PCMX had mutagenic effects on red blood cells of rainbow trout. Furthermore, when fish exposed to 4.2 ± 0.9 μg L^{-1} of PCMX for 40 days, CAT, HSC70A, HSP90BB, SOD, GPX2 gene expressions were down regulated while GPX1 and HSP90BA genes were up regulated and GSTA gene was unregulated. Epithelial lifting and edema in gills; degradation in hepatic cell nuclei and fat vacuoles in liver; cloudy swelling in the tubules, renal tubule epithelial cells degeneration, glomerular capillaries dilation, pycnotic nuclei in the hematopoietic tissue and glomerulus degeneration in kidney were observed. Thus, PCMX affect gene regulation and structure of the tissue and it can cause genotoxicity to red blood cells.

Methylisothiazolinone (MIT) and Methyichloroisothiazolinone (MCI) are often used in conjunction as preservatives in the different products (Yazar et al., 2011). MIT which is at a very low concentration can yield such an allergic response (Castanedo-Tardana and

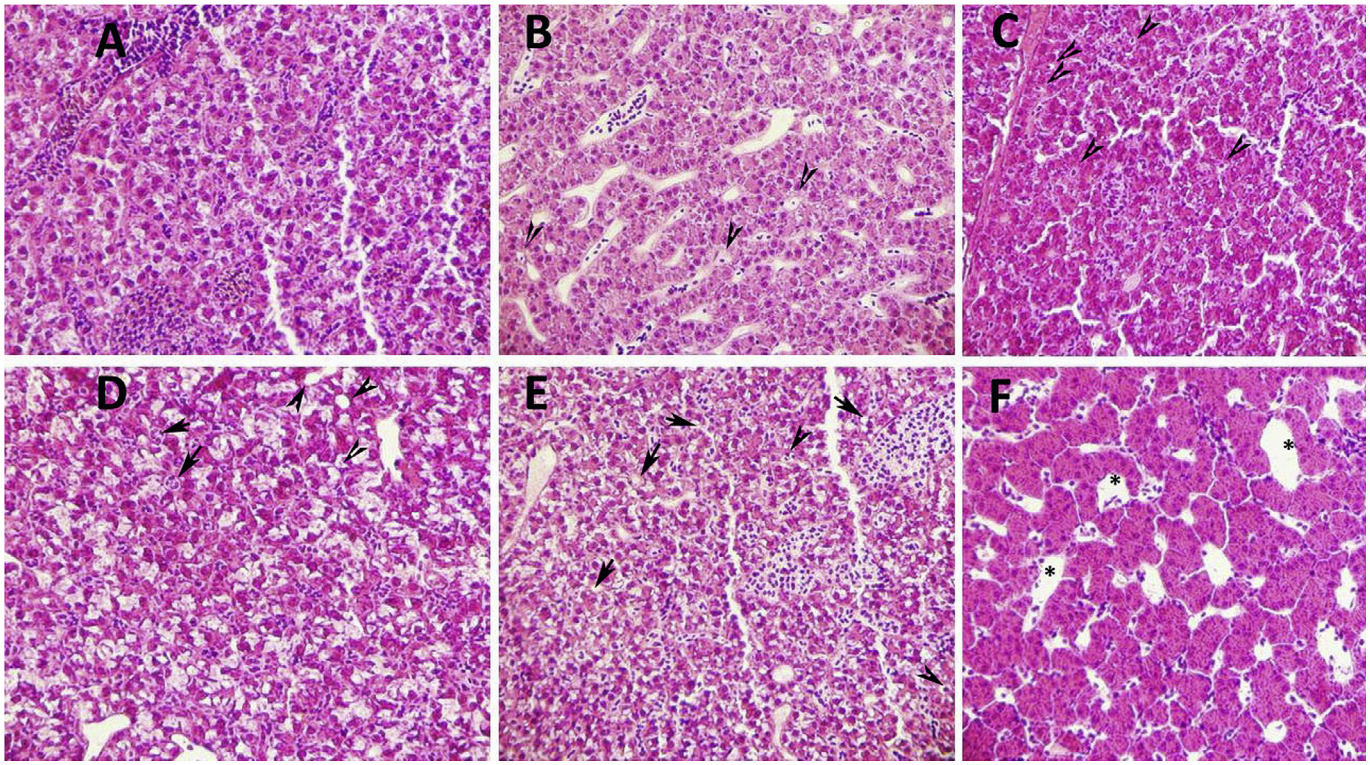

Fig. 3. Histopathology of rainbow trout liver stained with H&E 40 days after exposure to (a) water (control), (b) alcohol, (c) BRX, (d) PMCX, (e) MIT, and (f) TRC. Histopathologic lesions were pyknotic nucleus (arrow head), degradation in hepatic cell nuclei (arrow), fat vacuoles (*).

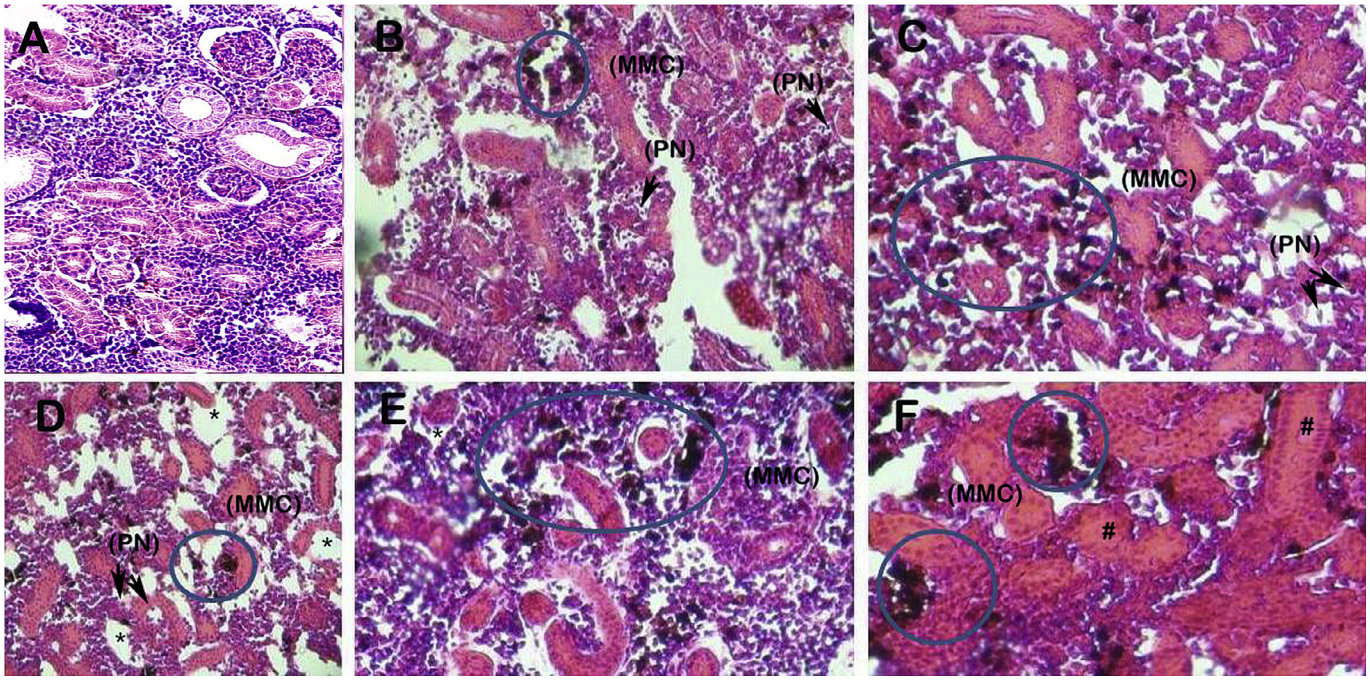

Fig. 4. Histopathology of rainbow trout kidney stained with H&E 40 days after exposure to (a) water (control), (b) alcohol, (c) BRX, (d) PMCX, (e) MIT, and (f) TRC. Histopathologic lesions were melanomacrophage centre (MMC), pyknotic nucleus (PN), glomerulus degeneration (*) and swelling tubules (#).

Zug, 2013). This ingredients being in low concentration does not always equate their safety, particularly when they are used continuously under low concentration (Boyapati et al., 2013). Current studies indicate that 100 ppm MIT in cosmetic products is not safe for the consumer (SCCS, 2014). MIT has been shown to be neurotoxic in vitro tests of neurons in mammalian tissue cultures and also a known environmental toxin, particularly to fish, though the majority of incidences are cause by non-cosmetic uses (Yazar

Table 4
The comet assay score in the red blood cell of fish exposed to chemical for 40 days. Treatment DNA damage scores (mean ± SD).

Treatment	DNA damage scores		
	Head Intensity	Tail Intensity	Tail DNA%
Negative control (dH_2O)	71.57 ± 11.38	28.43 ± 9.89	39.72
Negative control (dH2O + alcohol)	44.05 ± 8.37	55.95 ± 7.75	127.01
Positive control (H_2O_2, 30%)	24.18 ± 6.7	75.82 ± 11.1	313.56
TRC in alcohol	20.09 ± 7.44	79.91 ± 7.80	397.83
MIT	15.52 ± 6.66	84.48 ± 7.41	544.45
PCMX in alcohol	19.16 ± 9.89	80.84 ± 8.71	421.88
BRX	28.17 ± 11.43	71.78 ± 12.01	254.81

Table 5
Relative gene expression (fold) of different genes in the liver and kidney of rainbow trout following exposure to Triclosan (TRC), Chloroxylenol (PCMX), Methylisothiazolinone (MIT) and Borax (BRX) for 40 days.

Genes	Chemicals			
	PCMX	BRX	MIT	TRC
CAT	0.22 ± 0.03	0.96 ± 0.14	1.27 ± 0.19	0.56 ± 0.08
*GPX*1	593.79 ± 89.07	957.08 ± 143.56	209.72 ± 31.46	626.47 ± 93.97
GSTA	1.04 ± 0.16	200.10 ± 30.02	121.81 ± 18.27	360.79 ± 54.12
*HSC70*A	0.09 ± 0.01	134.64 ± 20.20	41.09 ± 6.16	172.58 ± 25.89
*HSP90*BA	19.83 ± 2.98	2129.69 ± 319.45	316.88 ± 47.53	640.62 ± 96.09
*HSP90*BB	0.08 ± 0.01	219.98 ± 33.00	84.45 ± 12.67	262.19 ± 39.33
SOD	0.19 ± 0.03	8.32 ± 1.25	17.45 ± 2.62	14.20 ± 2.13
*GPX*2	0.01 ± 0.00	629.72 ± 94.46	664.21 ± 99.63	786.10 ± 117.92

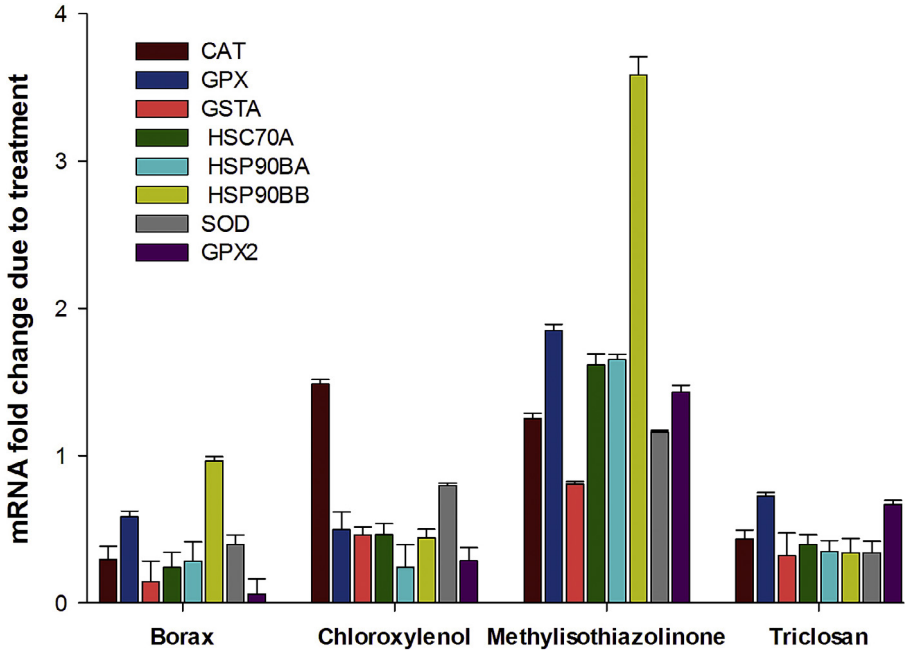

Fig. 5. Fold changes for mRNA expression of Cu/Zn-superoxide dismutase (*SOD*), glutathione peroxidase 1 (*GPX*1), glutathione peroxidase type 2 (*GPX*2), glutathione S-transferase A (*GSTA*), heat shock 90 kDa protein 1 beta isoform b (*HSP90*BB), heat shock 90 kDa protein 1 beta isoform a (*HSP90*BA), heat shock 70 kDa protein 8 isoform a (*HSC70*A) and catalase (*CAT*) in TRC, PCMX, MIT and BRX exposed rainbow trout. Fold changes were relative to the unexposed control group. Data represents means ± SEM.

et al., 2011). Developmental and chronic feeding/carcinogenicity studies in rats resulted in no significant effects (EPA, 1998). On the contrary, in the present study, MIT has the most genotoxic effects on fish than other chemicals used in this study, despite its use at very low concentrations. All genes studied in the present study were up regulated after exposing to MIT and most affected gene was GPX2 increased by 629.72 fold. In addition, recent regulations by the European Commission have banned the mixture of MCI and MIT in all leave-on body products (Yu et al., 2016).

It was known until now that BRX is of low acute toxicity and do not have any genotoxic or carcinogenic potential. Its toxicological effects at high doses are indicated by reversible histological effects on the testis and a reduction in fertility (HERA, 2005). On the other hand, recent studies have shown that borax has genotoxic effects on aquatic life based on BRX concentration and time dependent manner (Gulsoy et al., 2015). When they exposed zebrafish (*Danio rerio*) to different concentrations of BRX at different times, maximum increase in % tail of zebrafish red blood cells was seen at

96 h at 64 mg L^{-1} BRX concentration (Gulsoy et al., 2015). Similar to Gulsoy et al. (2015) study, in the present study, 8.9 ± 1.8 mg L^{-1} of BRX exposed fish for 40 days had red blood cells DNA damage. Borax concentrations in surface water range from <0.001 to 7 mg L^{-1} in different countries, with mean values typically below 0.6 mg L^{-1} (WHO, 1998). Topal et al. (2016) show that BRX can be toxic to rainbow trout and cause histopathological damage on gill, kidney and muscle of fish after acute exposure to BRX. Similar results were obtained in this study and significant histopathologic effects occurred in gill, spleen, liver, and kidney tissue of fish exposed to low concentrations of BRX. Furthermore, kidney is the most affected organ either after acute (Topal et al., 2016) or chronic (present study) exposure to BRX. All the studied genes except CAT were up regulated and most affected gene was *HSP90*BA increased by 2129.69-fold while least affected gene was CAT gene that was unregulated.

Comet assay made from fish erythrocyte can be used to monitor aquatic environments affected by effluents (SanJuan-Reyes et al., 2015). This assay is non-specific for mutagenic damage, thus it is possible to obtain false-positive results when assaying the genotoxicity of pollutants in situ (Bagdonas, 2007). Erythrocytes based comet assay can also be used to determine genotoxicity and putative mutagenic effects of benzene on electric fish *Apteronotus bonapartii* (Bücker et al., 2012). Ullah et al. (2016) determined the genotoxic effect of pesticide on blood erythrocytes of *Cirrhinus mrigala*. Demir et al. (2015) assayed for genotoxicity induced by nutrient pollution using the Comet assay in the peripheral blood cells of rainbow trout. Moreover, the results of our study have clarified the effect of antibacterial chemicals on the level of DNA damage in fish erythrocytes. According to the results of comet, borax is less harmful chemicals than others.

In conclusion, the results of this study suggest that TRC, PCMX, MIT can be toxic to fish and cause genotoxic effect on fish. In addition, BRX may have the same effect if fish are subjected to chronic exposure. Therefore, genotoxic, mutagenic and other potential effects of these compounds should be assessed in the aquatic environment. The monitoring of the remnants of these chemicals in the receiving waters is very important in terms of reducing the effects on the environment.

Present study indicates that the chronic toxic effect of TRC, PCMX, MIT, and BRX is high in rainbow trout. Therefore, usage and release of these chemicals to receiving water should be reduced. Furthermore, people who use these chemicals for cleaning should be more careful in order to protect aquatic environment.

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