Genomic markers for the biological responses of Triclosan stressed hatchlings of Labeo rohita

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Abstract

Triclosan (TCS) used commonly in pharmaceuticals and personal care products has become the most common pollutant in water. Three days old hatchlings of an indegenous fish, Labeo rohita were given 96h exposure to an environmentally relevant (0.06mg/L) and two moderately lethal concentrations (0.067 and 0.097 mg/L) of TCS and kept for 10 days of recovery for recording transcriptomic alterations in antioxidant/detoxification (SOD, GST, CAT, GPx, GR, CYP1a and CYP3a), metabolic (LDH, ALT and AST) and neurological (AchE) genes and DNA damage. The data were subjected to Principal Component Analysis (PCA) for obtaining biomarkers for the toxicity of TCS. Hatchlings were highly sensitive to TCS (96h LC 50 = 0.126mg/L and risk quotient = 40.95), 96h exposure caused significant induction of CYP3a, AChE and ALT but suppression of all other genes. However, expression of all the genes increased significantly (except for a significant decline in ALT) after recovery. Concentration dependent increase was also observed in DNA damage [Tail Length (TL), Tail Moment (TM), Olive Tail Moment (OTM) and Percent Tail DNA (TDNA)] after 96h. The damage declined significantly over 96h values at 0.06 and 0.067 mg/L after recovery, but was still several times more than control. TCS elicited genomic alterations resulted in 5-11% mortality of exposed hatchlings during the recovery period. It is evident that hatchlings of L. rohita are a potential model and PCA shows that OTM, TL, TM, TDNA, SOD and GR (association with PC1 during exposure and recovery) are the biomarkers for the toxicity of TCS.

1. Introduction

Triclosan [5-chloro-2-(2,4dicholorophenoxy) phenol] or TCS is commonly used as an antibacterial and antifungal agent in human medicines, personal care products (soap, toothpaste, mouthwash, detergents, handwash, deodorant, shampoo etc.) and household products (toys, cutting boards, containers, furniture etc.). It is also used as a veterinary medicine and a growth promoter for livestock and agricultural species (Daughton and Ternes 1999; Dann and Hontela 2011). In 2005, Halden and Paull reported that more than 3 million tons of TCS were being used in Europe and around 3.5 million tons were consumed in USA. Use of triclosan has been restricted in Europe (European Commission 2011), US (FDA 2016) and Canada (ECCC and HC 2016) now, but it is still widely used in other countries. Approximately 96% of the products that drain in sewage system have 0.1-0.3% of TCS by weight (Reiss et al. 2002), therefore it has become a common contaminant in water bodies all over the world (Singer et al., 2002; Sabaliunas et al. 2003; Miller et al. 2008). Concentration of TCS has been reported in the range of 1.4 - 40,000 ngL^{-1} in surface waters, 20 - 86161 ngL^{-1} in waste water influents, 23 - 5370 ngL^{-1} in waste water effluents, 0.001 - 100 ngL^{-1} in sea waters, 100 - 53,000 μg kg^{-1} dry weight in sediments of lakes and rivers, 0.02 - 35 μg kg^{-1} dry weight in sediments of marine waters, 20 – 133,000 μg kg^{-1} dry weight in biosolids of WWTPs, 580 – 15600 μg kg^{-1} dry weight in digested sludge and 0.201 – 328.8 μgL^{-1} in pore water from various parts of the world (Dhillon et al. 2015). In India also, high concentration of TCS has been reported in the water of Tamiraparani (0.944 μgL^{-1}), Kaveri and Velar (3.8 - 5.16 μgL^{-1}) rivers by Ramaswamy et al. (2011) and in sediments of Gomti River (5.11-50.36 μg/kg) by Nag et al. (2018).

Lipophillic nature, low Henry's constant (1.5×10^{-7} atm mol^{-1} m^{-3}) and high bioaccumulation factor (2.7 – 90) and log k_{ow} (4.8) seem to be responsible for decrease in volatilization and an increase in bioaccumulation potential of TCS (Ni et al. 2005; Dhillon et al. 2015). Along with it, aromatic nature and high chlorine content have been related to its lower degradation and higher persistence in the environment (Yueh and Tukey 2016). Its persistence in surface water and aerobic soils has been reported to be about 18 days (Bester 2005; Ying and Kookana 2007) and its high levels have been recorded in algae, crustaceans, shellfish, fishes, marine mammals and urine, blood, liver, adipose tissue, brain and breast milk of humans (Adolfsson-Erici et al. 2002; Allmyr et al. 2006; Heffernan et al. 2015). TCS is highly toxic to the organisms living in the aquatic environment particularly immediately downstream of the effluents from household wastewaters (Brausch and Rand 2011). Disturbance of metabolic pathways and hormone regulation (Sola- Gutierrez et al. 2018) by TCS and its toxic byproducts inturn induces oxidative stress, apoptosis, inflammation, diabetes and carcinogenesis (Ruszkiewicz et al. 2017). Generation and accumulation of ROS under the stress of TCS then regulates defensomes associated with detoxification, antioxidants and stress defense systems inturn lead to reduced life span and reproductive mechanisms.

TCS induced alteration in the expression of detoxification genes has been reported in *Chlamydomonas reinhardtii* (Pan et al. 2018), monogonont rotifer *Brachionus koreanus* (Han et al. 2016), copepod *Tigriopus japonicus* (Park et al. 2017), *Chironomus riparius* larvae (Martinez-Paz 2018), liver of *Bufo gargarizans* tadpoles (Chai et al. 2017), swordtail fish *Xiphophorus helleri* (Liang et al. 2013) and yellow catfish *Pelteobagrus fulvidraco* (Ku et al. 2014) and liver and kidney of rainbow trout, *Oncorhynchus mykiss* (Capkin et al. 2017). Variation in the toxicity of TCS to organisms in the previous reports demands more of such studies especially because toxicity varies with species, physiological state and environmental condition of the habitat of an organism (Tatrazako et al. 2004). Early life stages of fish are preferred for this purpose because these are easy to handle in a laboratory and more susceptible to stress due to premature body and weak defense system. At the same time, the results are directly applicable for humans because of genetic and physiological similarity of fish with mammals (DeMicco et al. 2010). Acute toxicity tests with mortality as an end point are traditionally used for evaluating impact of anthropogenic stress because of their simplicity but for establishing relationships between exposure stress and biological responses, there is a need to pay attention at the molecular level along with mortality.

DNA damage and rate of transcription are directly corelated to the production of metabolites for maintaining health, vitality and survival of an organism therefore we observed expression of the genes for phase I, phase II detoxification, antioxidant, metabolic and neurological enzymes and DNA damage along with mortality of the hatchlings of an indegenous food fish *Labeo rohita* after 96h exposure and 10 days post exposure (recovery) to TCS. We tried to find out biomarkers for the stress of an environmentally relevant (0.06mg/L) and two moderately lethal (0.067 and 0.097mg/L) concentrations of TCS. The study holds importance as it will help to improve our understanding of the relationship between stress and biological responses and will also help in regulatory submission of this emerging stressor in the environment and food fishes.

2. Materials And Methods

2.1 Experimental fish: Embryos/ fertilized eggs of *Labeo rohita* were procured from the Govt. Fish Farm Rajasansi, Amritsar and transported to the laboratory in oxygenated bags. After washing in 0.9% saline, the eggs were transferred to plastic tubs (8L capacity, n=50) filled with dechlorinated tap water (pH 7.3-7.9) for hatching under natural photoperiod (12:12h light-dark). After three days of hatching when whole yolk sac was completely absorbed, swim bladder developed, pigmentation appeared and active swimming started, the hatchlings were exposed to TCS.

2.2 Chemicals: Triclosan or Irgasan (CAS ID 3380-34-5) was purchased from Sigma Aldrich, USA, (purity >97%). A stock solution of 10mg/ml was prepared using acetone as solvent. All the chemicals used in the study were AR grade.

2.3 Determination of 96h LC_{50}: Semistatic bioassays (daily bath replacement) were conducted according to the OECD guidelines 210, Fish early-life stage toxicity test (1992), to find out 96h LC_{50} value. Test water was changed 1h after feeding the hatchlings with boiled egg yolk. Range finding bioassays were conducted to find out 96h LC_0 and LC_{100} values for the hatchlings. Hatchlings were then exposed in 1L plastic jars in triplicate (n=10/replicate) to different concentrations of TCS between the LC_0 – LC_{100} values. Mortality was recorded at 24h intervals through 96h and dead hatchlings were removed from the jars immediately, when noticed. A hatchling that did not move on prodding with a rod was considered dead.

2.4 Gene expression and Genotoxicity studies: 120 hatchlings were exposed in sextuplicate (n=20/replicate) to control (tap water), solvent control (acetone), LC_0 (0.06mg/L), LC_{10} (0.067mg/L) and LC_{30} (0.097mg/L) for 96h. After the exposure, some of the hatchlings from each concentration were used for transcriptomic profiling and single cell gel electrophoresis (comet assay) and rest of the larvae were kept in tap water for a recovery period of 10 days. Same parameters were recorded at the end of recovery period to observe any reversal or prolongation of the toxic effects of the selected concentrations of TCS.

2.4.1 Primer Design: Primers of the target genes Cytochrome P_{450}1A (CYP1A), Cytochrome P_{450}3A (CYP3A), Glutathione-S- Transferase (GST), Cu/Zn Superoxide Dismutase (Cu/Zn SOD), Catalase (CAT), Glutathione Reductase (GR), Glutathione peroxidase (GPx), Lactate Dehydrogenase (LDH), Aspartate Transaminase (AST), Alanine Transaminase (ALT), Acetylcholine esterase (Ach) and housekeeping / reference gene, Beta–Actin (β-actin) were designed according to the mRNA sequences from NCBI and EMBL Databases using Primer 3 software . Table 1 shows the primer sequences along with their annealing temperatures (Tm) and accession number.

2.4.2 Total RNA isolation and Reverse Transcription: Total RNA was isolated from the larvae using Trizol reagent (Invitrogen) according to manufacturer's instructions. The extracted RNA was treated with DNAase (Biolabs) to eliminate DNA contamination. Quality and purity of the isolated RNA was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Integrity of RNA was checked by agarose gel electrophoresis and then 1µg of mRNA was reverse transcribed to cDNA by using Biorad mRNA to cDNA preparation kit. The prepared cDNA was further diluted 10 folds and used for expression profiling.

2.4.3 Quantitative Real Time PCR (qRT-PCR): The qRT-PCR was performed in three biological and technical replicates on Biorad CFX-manager 3.1, using Kapa biosystems SYBR green Mastermix. The reaction mixture (10µ1) contained 1µ1 cDNA, 5µ1 SYBR green Mastermix, 0.15µ1 (5µM F+R) primer and 3.85µ1 RNase free water. Reaction was performed at the following thermocycling conditions: initial denaturation at 95^0C for 10 min, 40 cycles of 15 sec at 95^0C, 40 sec of annealing and finally 1 min extension at 60^0C. The melting curve analysis was performed to ensure amplification of the specific amplicon. The results of gene expression were analysed according to the method of Livak and Schimittgen (2001). The normalized expression or relative fold change at the expression level was represented as .

2.5 Genotoxic Effect: Genotoxic effect of TCS was evaluated with the help of comet assay (Single Cell Gel Electrophoresis) according to Singh et al. (1988) with slight modifications.

2.5.1 Sample preparation: Cell suspension was prepared by homogenizing live hatchlings in ice cold phosphate buffer saline (pH=7.4) in triplicate. The homogenate was centrifuged at 200g for 15 min at 4^0C, the supernatant was discarded and the pellet was resuspended in buffer and centrifuged again. This step was repeated 2-3 times and finally the pellet was resuspended in 100µl of PBS and used for comet assay.

2.5.2 Lysis Buffer: Stock solution of the lysis buffer (445ml, pH 10) was prepared by adding 73.01g NaCl, 18.7g EDTA, 0.6g Tris and 4g NaOH in distilled water. Working solution was prepared by adding 44.5ml of DMSO and 4.95ml of Triton X to the stock solution.

2.5.3 Electrophoresis Buffer: Stock solution of NaOH (40g) and EDTA (7.44g) was prepared separately in 100ml of double distilled water. The working solution contained 965ml of chilled double distilled water, 30ml of NaOH and 5ml of EDTA.

2.5.4 Tris Buffer: Tris buffer was prepared by adding 4.845g of Tris buffer in 100ml of double distilled water.

2.5.5 Slide Preparation and electrophoresis: Base layer of 1% Normal Melting Point Agarose (NMPA) was applied on the slide and it was kept for 12h before the second layer of 35µl cell suspension + 120µl of 0.5% Low Melting Point Agarose (LMPA) and the top layer of 0.5% LMPA were applied one after the other. The slide was covered with a coverslip and kept at 4^0C for 15-20 min for gel casting. The slide was dipped in lysis buffer for 2h at 4^0C in dark. After lysis, the slide was immersed in electrophoresis tank containing electrophoresis buffer and run at 300 mA and 20V for 20 min. The slide was neutralized in Tris buffer. Then the slide was washed with chilled distilled water, stained with ethidium bromide and observed under Nikon ECLIPSE E200 Fluorescent microscope and photographed with Nikon D5300 camera. For each treatment 300 cells were scored by Casp Lab Software for Tail length, Tail moment, Olive Tail moment and % Tail DNA to evaluate the extent of DNA damage.

2.6 Statistical analysis: Probit analysis (Finney 1971) was performed to find out 96h LC_{50} value and 95% confidence limits using SPSS 16.0 Software. One way Analysis of Variance (ANOVA) was performed to find out differences among the means. Tukey's test (between the treatments) and Student's t-test (between the durations) were used to determine the level of significance at 5% ($p \leq 0.05$) and 1% ($p \leq 0.01$). Data have been represented as Mean ± Standard deviation. Principal component analysis (PCA) was performed for obtaining relationship between the biomarkers.

3. Results

3.1 Toxicity of TCS: The 96h LC_0 and LC_{100} of TCS were observed at 0.06 and 0.26 mg/l TCS, respectively while 96h LC_{50} and its 95% fiducial limits were calculated at 0.126mg/L and 0.11-0.13 mg/l TCS, respectively (Table 2). A concentration dependent increase was observed in percent mortality of hatchlings during the bioassay. After 72h, 3-5% of TCS exposed hatchlings became paralysed, showed slight movement, ate very less and had thin body compared to the control hatchlings. Paralysed hatchlings generally remained at the bottom but showed occasional erratic circular swimming.

Average weight of control and 0.097 mg/L TCS exposed larvae were 1.37 and 1.29 mg, respectively after 96h and 3.1mg and 2.8mg, respectively after recovery. During the recovery period, corrected mortality was 5, 7 and 11% at $LC_{0,}$ $LC_{10,}$ and LC_{30} concentrations, respectively.

3.2 Transcriptomic profiling:

3.2.1 CYP1A: Fig 1a shows that level of CYP1A mRNA declined significantly ($p \leq 0.01$) at 0.06 and 0.067mg/L but increased significantly ($p \leq 0.01$) over control at the highest concentration (0.51 folds). This was followed by a rise ($p \leq 0.01$) over control at all the concentrations after the recovery period. Compared to the 96h values increase was maximum at 0.067mg/L (3.48 folds) and minimum at 0.097mg/L (1.04 folds).

3.2.2 CYP3A: Fig. 1b highlights that there was a significant ($p \leq 0.05$) concentration dependent induction of CYP3A expression over control after the exposure as well as the recovery period. After the recovery period, the expression of CYP3a decreased non-significantly over 96h values at 0.067 and 0.097 mg/L (0.66 and 1.25 folds respectively) but increased non-significantly at 0.06mg/L (0.26 folds).

3.2.3 GST: Fig. 1c shows that a significant ($p \leq 0.01$) concentration dependent decline over control in the expression of GST after 96h exposure was followed by a significant ($p \leq 0.01$) increase (0.90, 0.70 and

3.80 folds at 0.06, 0.067 and 0.097mg/L respectively) over control after 10 days of the recovery period. After recovery, expression of this gene increased over 96h values but it was significant ($p \leq 0.05$) at 0.067 and 0.097mg/L only (1.15 and 4.27 folds, respectively).

3.2.4 SOD: Fig. 1d depicts that after 96h exposure, there was a significant ($p \leq 0.01$) decline over control in the expression of SOD gene. However, the trend of expression of SOD gene turned opposite after the recovery period, where a significant ($p \leq 0.01$) increase over control was observed at all the concentrations. After recovery, expression of SOD was significantly ($p \leq 0.05$) elevated over the respective 96h values at 0.06 (1.03 folds), 0.067 (4.61 folds) as well as 0.097 mg/L (1.95 folds).

3.2.5 CAT: Expression of CAT was non-significantly less than control at the two lower concentrations of TCS but it was significantly ($p \leq 0.01$) elevated at the highest concentration (1.82 folds), after 96h exposure (Fig. 1e) This was followed by a significant increase over control ($p \leq 0.05$) at all the concentrations after the recovery period. On this day, significant ($p \leq 0.05$) increase was also observed over 96h values at the two lower concentrations but there was a nonsignificant decline at 0.097mg/L**.**

3.2.6 GR: GR expression showed a significant ($p \leq 0.01$) decline over control after 96h exposure to TCS and was followed by a significant ($p \leq 0.01$) induction after the recovery period (Fig. 1f.). Increase was maximum at 0.067mg/L (32.11 folds), intermediate at 0.097mg/L (14.07 folds) and minimum at 0.06mg/L (7.0 folds). After recovery, the values were 7.67. 32.96 and 14.80 folds more than 96h values ($p \leq 0.05$) at 0.06, 0.067 and 0.097mg/L, respectively.

3.2.7 GPx: Fig. 1g depictes a significant ($p \leq 0.01$) concentration dependent decline in the expression level of GPx on exposure to TCS. However, there was a significant ($p \leq 0.05$) induction of GPx expression over control, after the recovery period. On this day, increase over 96h values was significant ($p \leq 0.05$) at 0.067mg/L (1.14 folds) and 0.097mg/L (1.30 folds) only.

3.2.8 LDH: Fig. 2a highlights that after 96h exposure, level of LDH mRNA showed significant ($p \leq 0.01$) reduction over control at 0.06 and 0.067 mg/L (0.74 and 0.83 folds, respectively) but a significant ($p \leq 0.01$) elevation at 0.097mg/L (0.52 folds). This was followed by a non-significant rise in LDH expression at all the concentrations after the recovery period. Increase in the expression of LDH over 96h was significant ($p \leq 0.05$) only at 0.067 mg/L (1.80 folds) after recovery.

3.2.9 AST: The significant ($p \leq 0.01$) concentration dependent reduction in the expression level of AST on exposure to TCS was followed a significant ($p \leq 0.05$) increase over control as well as 96h values after the recovery period (Fig. 2b). The increase over 96h values was 3.65, 2.21 and 4.12 folds more at 0.06, 0.067 and 0.097mg/L, respectively.

3.2.10 ALT: ALT mRNA copy number was significantly ($p \leq 0.01$) elevated after 96h exposure in a concentration dependent manner. This was followed by concentration dependent decline ($p \leq 0.01$) during the recovery period and the values at 0.06, 0.067 and 0.097mg/L were significantly ($p \leq 0.05$) less (1.27, 3.36 and 4.93 folds, respectively) than the respective 96h values also (Fig. 2c).

3.2.11 Ache: Fig.2d shows that there was a significant ($p \leq 0.05$) rise over control in the expression level of acetylcholine esterase during the exposure as well as the recovery period. After recovery, elevation at 0.06, 0.067 and 0.097mg/L (0.63, 0.05 and 0.75 folds, respectively) was non-significantly more than the respective 96h values.

3.3 Comet Assay: There was a significant ($p \leq 0.01$) concentration dependent increase over control in TL, TM, OTM and % TDNA after 96h exposure as well as the recovery period. Although after recovery, all the parameters declined compared to 96h exposure but the decline was significant ($p \leq 0.05$) for TL and TM only at 0.06 and 0.097mg/L and for % TDNA at 0.06mg/L. Maximum decline was observed in TM while minimum decline was observed in OTM (Fig. 3, 4).

3.4 Principal component analysis (PCA): PCA generated two principal components after 96h (Eigen value > 1) but generated three principal components after 10 days of the recovery period with total variance of 95.40% and 95.68%, respectively (Table 3). After 96h exposure, PC1 accounted for 64.25% of total variance and showed association with OTM, ALT, GR, TL, TM, CYP3A, SOD, TDNA, GPx and AST. PC2 included LDH, CAT, CYP1A, GST and AChE and accounted for 31.15% of total variance. After 10 days of the recovery period, PC1 accounted for 37.80% of the total variance and showed association with CAT, GR, SOD, TL, OTM, TM and TDNA while AChE, AST, GPx and GST were associated with PC2 and accounted for 33.47% of total variance. PC3 showed association with CYP3A, LDH, CYP1A and ALT and accounted for 24.41% of total variance.

4. Discussion

The hatchlings of *L. rohita* were observed to be highly sensitive to the stress of TCS as the value of 96h LC_{50} and LC_{100} was 0.126mg/L and 0.26 mg/L, respectively. The risk quotient (RQ) of TCS for the larvae came out to be 40.95 (EC 2003). As per the standard evaluation procedure of USEPA for fresh water fish, a chemical having 96h LC_{50} < 1mg is highly toxic (Zucker 1985) and RQ value > 1 indicates high risk and sensitivity of the organisms (Hernando et al. 2006). Sensitivity of fish to the stress of a toxicant generally varies with age, size and species (Eaton and Gilbert 2008) and early developmental stages are vulnerable than other stages (Dann and Hontela 2011). This is clearly evident from the comparison of the earlier reports for 96h LC_{50} value of TCS for the embryos of *Oryzias latipes* (0.169 mg/L), *Cyprinus carpio* (0.315 mg/L), *Ctenopharyngodon idella* (0.116 mg/L), *Cirrhinus mrigala* (0.131mg/L), *L. rohita* (0.096mg/L) by Horie et al. (2018) and Dar et al. (2019), early life stages or larvae of Japanese medaka (0.6 and 0.118 mg/L) by Ishibashi et al. (2004) and Horie et al. (2018), respectively, fingerlings of *L. rohita* (0.39mg/L) by Hemalatha et al. (2019) and adult fish [*Lepomis macrochirus* (0.37 mg/L), *Pimephales promelas* (0.26mg/L) and *Xiphophorus helleri* (1.47 mg/L)] by Orvos et al. (2002) and Liang et al. (2013). TCS affects excitation- contraction of skeletal muscles (Fritsch et al., 2013), this could have resulted in abnormal swimming by the hatchlings in the present study. Alteration of swimming behaviour in TCS exposed larvae of rainbow trout and fathead minnow has also been observed by Orvos et al. (2002) and Fritsch et al. (2013).

Involvement of ROS is confirmed in the mechanism of TCS action therefore the underlying cause for the observed concentration dependent mortality of the larvae in the present study seems to be the TCS induced oxidative stress. This was also evident from the suppression of the expression of antioxidant, detoxification and metabolic genes as well as an increase in DNA damage. During oxidative stress, the balance between generation and destruction of ROS is disturbed which leads to further production and accumulation of ROS in the body. This results in DNA damage, depletion of antioxidants, lipid peroxidation and cellular damage (Kim et al. 2020) which gradually cause morbidity or mortality of organisms. Expression of antioxidant enzymes and extent of DNA damage therefore reflect not only the degree of toxicity of a chemical but also the ability of an organism to tolerate the oxidative stress (Puckette et al. 2007; Zhou et al. 2009).

Cytochrome P450 (CYP) is a superfamily of heme containing monooxygenases which play a crucial role in phase1 biotransformation of xenobiotics (Chaty et al. 2004; Dejong and Wison 2014). A significant increase in the expression of CYP3a at all the concentrations and that of CYP1a at the highest concentration of TCS indicates that TCS is an inducer of these genes. CYP enzymes are usually regulated by specific receptors and the pollutants having affinity for the chemical structure of these receptors are predominately responsible for the altered gene expression. Aryl hydrocarbon receptor (AhR), regulator of the activity of a diverse set of genes including CYPs (Kawajiri and Fujii- Kuriyama 2007) is a ligand activated transcription factor. Expression of AhR regulated genes is generally modulated by polycyclic aromatic hydrocarbons, polyphenolics and dioxins which have an affinity for AhR (Sarkar et al. 2006; Liang et al. 2013; Szychowski et al. 2016). TCS is an aromatic compound and is converted to dioxins in the presence or absence of sunlight during the exposure as well as the recovery period therefore it may have activated AhR to induce transcription of CYP genes of the larvae (Guengerich et al. 2003). Induction of CYP1a and CYP3a mRNA has been reported in swordtail fish and CD-1 and C57BL/6 mice after TCS exposure by Liang et al. (2013) and Wang et al. (2017). Due to involvement of ROS, interaction of Nrf2/Keap1 transcription factor and HIF-α with AhR of the hatchlings can not be ignored (Haarmann-Stemmann et al. 2012). These sensitive proteins undergo reversible oxidative and reductive reactions and activate or delay downstream signaling pathways including antioxidant enzyme system (Hertog et al. 2005; Liu et al. 2005) which generally counteracts the accumulation of ROS for prevention of cellular damage in stressed organisms (Valavanidis et al. 2006). In addition to this Jacobs et al. (2005) reported that TCS has moderate affinity for the human Pregnane X receptor (another ligand activated transcription factor) that also plays an important role in regulation of CYP3a family of genes (Hollenberg 2002; Wassmur et al. 2010).

GST (Phase II detoxifying enzyme) is known to generate less toxic and more hydrophylic molecules for preventing oxidative damage by conjugating the breakdown products of peroxidases to GSH (Olsen et al. 2001; Fernendes et al. 2008). GST proteins display a broad substrate specificity and their expression can be modulated by exogenous compounds as well as the compounds oxidized by CYP450 enzymes (Martinez-Paz 2018). GST acts as a transport protein during detoxification processes which could be the reason for the observed concentration dependent decline in the expression of GST after 96h exposure and an increase during the recovery period. The lesser increase in CYP3a during the recovery period could have required lesser conjugation and transportation reaction and as a result higher level of GST was observed. Downregulation of GST genes by TCS during 96h exposure can also be related to its structural similarity with polychlorobiphenols which are potent inhibitors of Phase II enzymes (Wang et al. 2004). In previous studies also, TCS has been reported to downregulate expression of GSTd6 (after 1000 µg/L for 24hr) in *Chironomus riparius* (Martinez- Paz 2018) and GST mRNA (after 500 µg/L for 168hr) in yellow catfish (Ku et al. 2014).

A concentration dependent decrease in the expression of SOD, CAT, GPx, GR, LDH and AST along with GST after 96h exposure to TCS in the present study also indicated that stress exceeded the capacity of the intrinsic antioxidant system of the larvae. ROS have been reported to cause repression of transcription by damaging the structure of enzymes (Slanninova et al. 2009). Modulations in these enzymes are considered an index for understanding toxicity mechanisms of a variety of xenobiotics (Regoli and Principato 1995) as well as an organism's ability to tolerate oxidative stress (Pukette et al. 2007; Zhou et al. 2009). The early life stages of fish do not have fully developed metabolic pathways to degrade xenobiotics (Embry et al. 2010) and inturn it resulted in strongly modulated gene expression during the recovery period. At lower levels, ROS have been shown to activate the pathways that reinforce defense responses and enhance survival to subsequent oxidative stress (Sanchez et al. 2015). SOD is the most active enzyme of this system, it catalyzes dismutation of superoxide (O_2^-) to O_2 and H_2O_2. H_2O_2 is further detoxified by CAT, GPx and GR to H_2O and O_2 (Song et al. 2006; Sun et al. 2009). Similar pattern of declined SOD expression has also been reported in male juvenile zebra fish (Wang et al. 2019a), gill and ovary of zebra fish (Wang et al. 2019b) and liver of tadpole *Bufo gargarizans* (Chai et al. 2017) after TCS exposure. The decrease during exposure in the present study was followed by an increase in expression of all the genes during the recovery period but the increase was 2-5 folds more than control.

CAT is responsible for preventing oxidation of unsaturated fatty acids in the cell membrane, therefore higher transcription level of this gene at 0.097mg/L TCS after 96h and at all the concentrations during the recovery period could be attributed to the production/ accumulation of pro-oxidants such as H_2O_2. An increase in the expression of CAT has been reported in TCS exposed *Brachionus koreanus* (Han et al. 2016) and *Chlamydomonas reinhardtii* (Pan et al. 2018). GR plays an important role along with GPx for maintenance of redox potential and is responsible for regeneration of glutathione during detoxification of peroxides and free radicals. Decline in the expression of both these genes after 96h exposure is supported by earlier studies which also reported a declined expression of Phospholipid hydroperoxide glutathione peroxidase (PHGPx) in liver of tadpole Bufo gargarizans (Chai et al. 2017), GPX4b in male juvenile zebra fish (Wang et al. 2019a), GPX1a in gill and ovary of zebra fish (Wang et al. 2019b). Significant inhibition of GR expression in the TCS exposed larvae in the present study is corroborated by the finding of Falisse et al. (2017) and Gyimah et al. (2020) who also reported declined GR activity in zebrafish early life stages and in the liver of adult zebra fish, respectively.

LDH is an important glycolytic enzyme responsible for energy production during anaerobic conditions and plays a very important role to meet the energy demand during stress conditions (Tseng et al. 2008). A significant increase in the expression of LDH at 0.097mg/L TCS in the present study indicated shift towards anaerobic metabolism of glycogen and other stored metabolites as suggested by Ellis (1937). An increase in lactic acid production and LDH-A expression has been related to increased energy requirement under the stress of TCS in HepG2 cells by An et al. (2020). Similarly, increased ALT level in the larvae during 96h exposure might have been due to increased transamination rates for increasing energy production through citric acid cycle in the TCS stressed larvae of *L. rohita*. An increased ALT expression has been reported by Fu et al. (2020) in zebra fish exposed to 400μg/L methyl triclosan for 96h. Decrease in the expression of AST but an increase in the expression of ALT in the present study indicates that TCS damaged liver of the larvae as the higher ratio of ALT:AST indicates liver damage. The decline in ALT expression but an increase in AST during the recovery period represents the induction of repairing mechanisms. This was also evident as food avoidance behavior and low body weight of the TCS exposed larvae till the end of recovery period.

TCS induced transcriptional changes are also common in brain (Haggard et al. 2016) and acetylcholine esterase is an important biomarker for determining neurotoxicity. This enzyme plays a key role in regulation of neuronal and muscular development and apoptosis (Hanneman, 1992; Behra et al. 2002). A concentration dependent increase in the expression of AchE during the exposure and its continuation during the recovery period seems to be responsible for the observed abnormal body posture and swimming behaviour of the larvae. Oliveira et al. (2009) and Falisse et al. (2017) also observed elevated AchE activity in zebrafish larvae (0.25mg/L TCS) and adult zebra fish (50 and 100μg/L TCS), respectively.

Genotoxic effect of TCS was observed to prolong till the end of recovery period. The concentration dependent increase in Tail length, Tail moment, Olive Tail moment and percent Tail DNA after 96h was followed by a small decline during the recovery period but the values were still 1.5- 2 folds more than control. The genotoxic effects of TCS have also been reported by Binelli et al. (2009), Lin et al. (2012) and Hemalatha et al. (2019) in zebra mussels, earthworms and fingerlings of *L. rohita,* respectively. TCS induces ROS production that act as reductants or oxidants and cause deleterious effects on DNA (Gniadecki et al. 2001). Higher level of CYPs in the exposed larvae can also be related to the concentration dependent increase in DNA damage as the CYPs are known to catalyze the formation of genotoxic or mutagenic intermediates from a number of polycyclic aromatic hydrocarbons (Shimada et al. 1996). The results of the comet assay in our study are supported by Gyimah et al. (2020) who also recorded similar effects of TCS on the liver cells of zebra fish.

Association of OTM, TL, TM, TDNA, CYP3a, SOD, GR, GPx, ALT and AST with PC1 after 96h exposure indicates that inspite of TCS inflicted DNA damage, the antioxidative/detoxification/metabolic genes tried to overcome the stress. However, during the recovery period CAT also showed association with PC1 for metabolizing the pro-oxidants that may have accumulated in the hatchlings during exposure. Based on the degree of similarity OTM, TL, TM, TDNA, SOD and GR could be ascertained as the biomarkers for the stress of TCS.

Conclusion: It seems that transcriptomic alterations and DNA damage collectively contributed to the observed concentration dependent mortality, feed avoidance and abnormal swimming in the hatchlings during exposure as well as the recovery period. The results clearly show that differential level of expression of different groups of genes is a useful source of biomarkers for evaluating toxicity mechanisms responsible for mortality under the stress of xenobiotics. The work also indicates possible use of the larvae of this indegenous species as a potential model for assessing the impact of TCS on the health and survival of aquatic organisms.

Declarations

Ethical Approval: This work deals with the larval stage of food fish and the food fishes do not come under the preview of animal ethics committee in India.

Consent to Publish: All the authors agreed to publish this work in ESPR.

Consent to Participate: Not Applicable.

Availability of data and materials: Data and materials are available with Corresponding author.

Conflict of Interest: The authors declare that there is no conflict of interest.

Author contributions: A.K. and S.S. designed study, S.S, O.I.D. and K.S. performed the experiments, S.T. and A.K.K helped in RT-PCR, A.K. and S.S. drafted the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1: Primer sequences, annealing temperatures and accession numbers of various genes used for RT-PCR.

Gene	Primer sequence 5’-3’	Tm	Accession number
CYP 1a	F: 5’TGAACATGAGCGACGAGTTC 3’ R: 5’TCGCAGAGGTTGATGAGAGA 3’	54 ^{0}C	DQ211095.1
CYP3a	F: 5’CAGCGAGGAACACACAGAAA 3’ R: 5’CCGGAGCCTTATTAGGGAAG 3’	53^{0}C	GU046696.1
GST	F: 5’AGCGGATGTTTGAGACCAAC 3’ R: 5’AAGTTCTTGCCAGCGAGGTA 3’	50 ^{0}C	EU107283
Cu/Zn SOD	F: 5’AAACTCACGGTGGACCAACT 3’ R: 5’ATTGCCTCCCTTACCCAAGT 3’	50^{0}C	CKX650370.1
CAT	F: 5’TCCACTCTCAGAAGCGGAAT 3’ R: 5’CTGAGCGTTGACCAGTTTGA 3’	54 ^{0}C	FJ560431.2
GR	F: 5’GCTGTGGAGATGGCTGGTAT 3’ R: 5’AACACTGAGGCCCTTGTCAG 3’	55 ^{0}C	HQ174244.1
GPx	F: 5’ATCAGGGCCTTGTGATTCTG 3’ R:5’CACAAACAGAGGGTGGGTTT 3’	51^{0}C	KX650369.1
LDH	F: 5’ACGCAAGCCTCTTTCTCAAG 3’ R:5’ATGCAGTTGGGGCTGTACTT 3’	50^{0}C	KC887544.1
AST	F-5’CCTTACCATGGACACCCTGA 3’ R-5’CCTGTCGAAGGAAGGTGATT 3’	50 ^{0}C	KC816542.1
ALT	F: 5’GGAGAAGTGCTGGAGTCTGG 3’ R: 5’TACACCTCGTCAGCCATCAG 3’	57 ^{0}C	AB911122.2
Ach	F: 5’GCTAATGAGCAAAAGCATGTGGGCTTG 3’ R: 5’TATCTGTGATGTTAAGCAGACGAGGCAGG 3’	53^{0}C	NP_571921
β-actin	F: 5’ AGGACCTGTATGCCAACACT 3’ R: 5’ GAACCTCCGATCCAGACAGA 3’	53^{0}C	EU184877.1

Table 2: 96h LC_0, LC_{50} and LC_{100} values of TCS for the hatchlings of *L. rohita.*

H	96h LC_{50} (mg/l)	96h LC_0 (mg/l)	96h LC_{100} (mg/l)	Fiducial range (mg/l)	Regression equation (Y = mx + c)	R^2	χ^2 value
ta	0.126	0.06	0.26	0.11-0.13	y = 417.1x - 6.071	0.951	38.580

Table 3: Statistical parameters generated by PCA for biomarkers in the hatchlings of *L. rohita* after 96h of exposure to TCS and 10 days of the recovery period.

Treatment	Component	Variable	Factor loading	Eigen value	% Variance	Cumulative %
96 h	**PC1**	OTM	0.98	10.70	64.25	64.25
		ALT	0.98			
		GR	0.98			
		TL	0.96			
		TM	0.96			
		CYP3A	0.96			
		SOD	0.95			
		TDNA	0.93			
		GPx	0.84			
		AST	0.79			
	PC2	LDH	0.99	3.61	31.15	95.40
		CAT	0.97			
		CYP1A	0.96			
		GST	0.78			
		AChE	0.76			
10 days	**PC1**	CAT	0.93	8.42	37.80	37.80
		GR	0.92			
		SOD	0.92			
		TL	0.88			
		OTM	0.83			
		TM	0.76			
		TDNA	0.70			
	PC2	AChE	0.98	3.23	33.47	71.27
		AST	0.89			
		GPx	0.88			
		GST	0.85			
	PC3	CYP3A	0.95	2.70	24.41	95.68
		LDH	0.92			
		CYP1A	0.81			
		ALT	0.61			

Figures

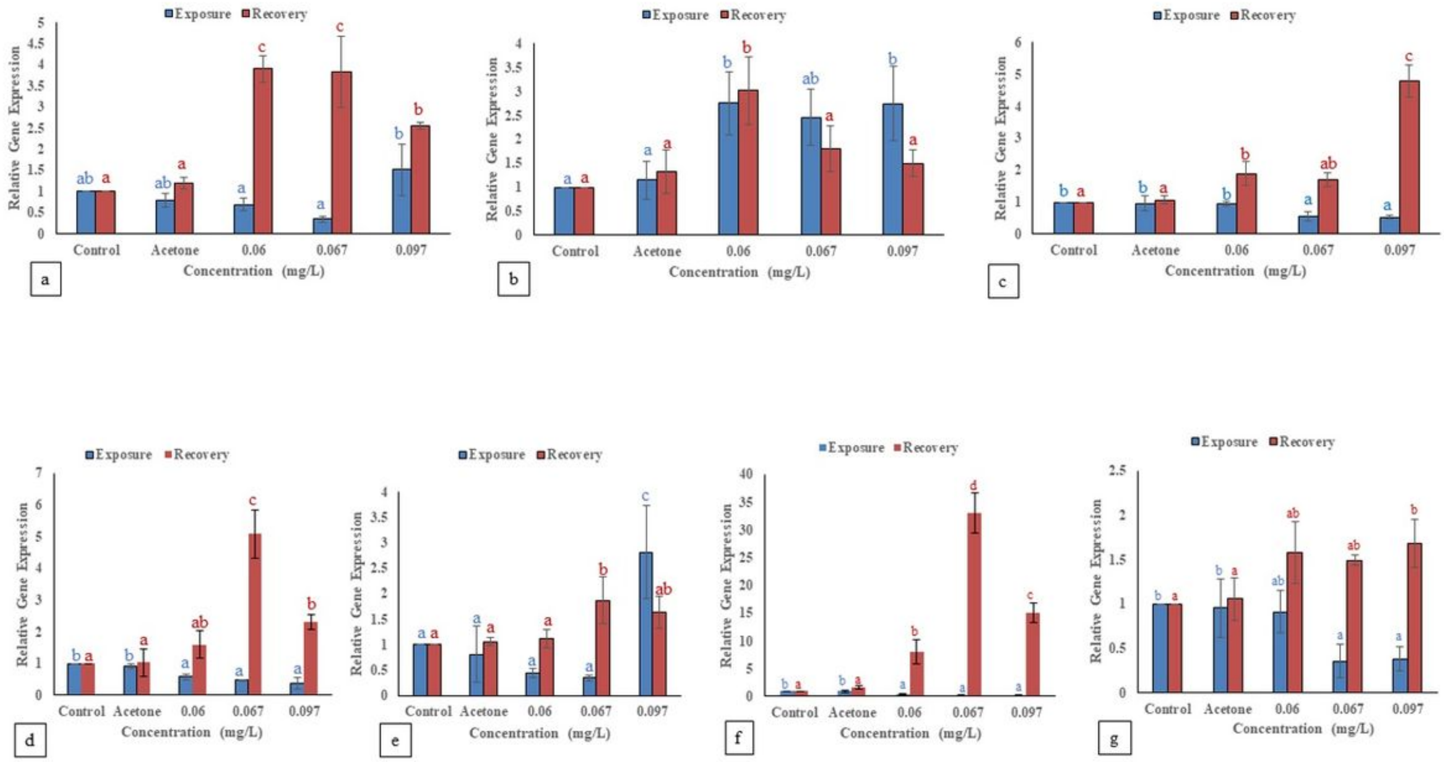

Figure 1

Effect of TCS on the expression of CYP1a (a), CYP3a (b), GST (c), SOD (d), CAT (e), GR (f) and GPx (g) in the hatchlings of L. rohita. Values are mean ± SD, n=3 and different alphabets represent significant (P≤0.05) difference among treatments.

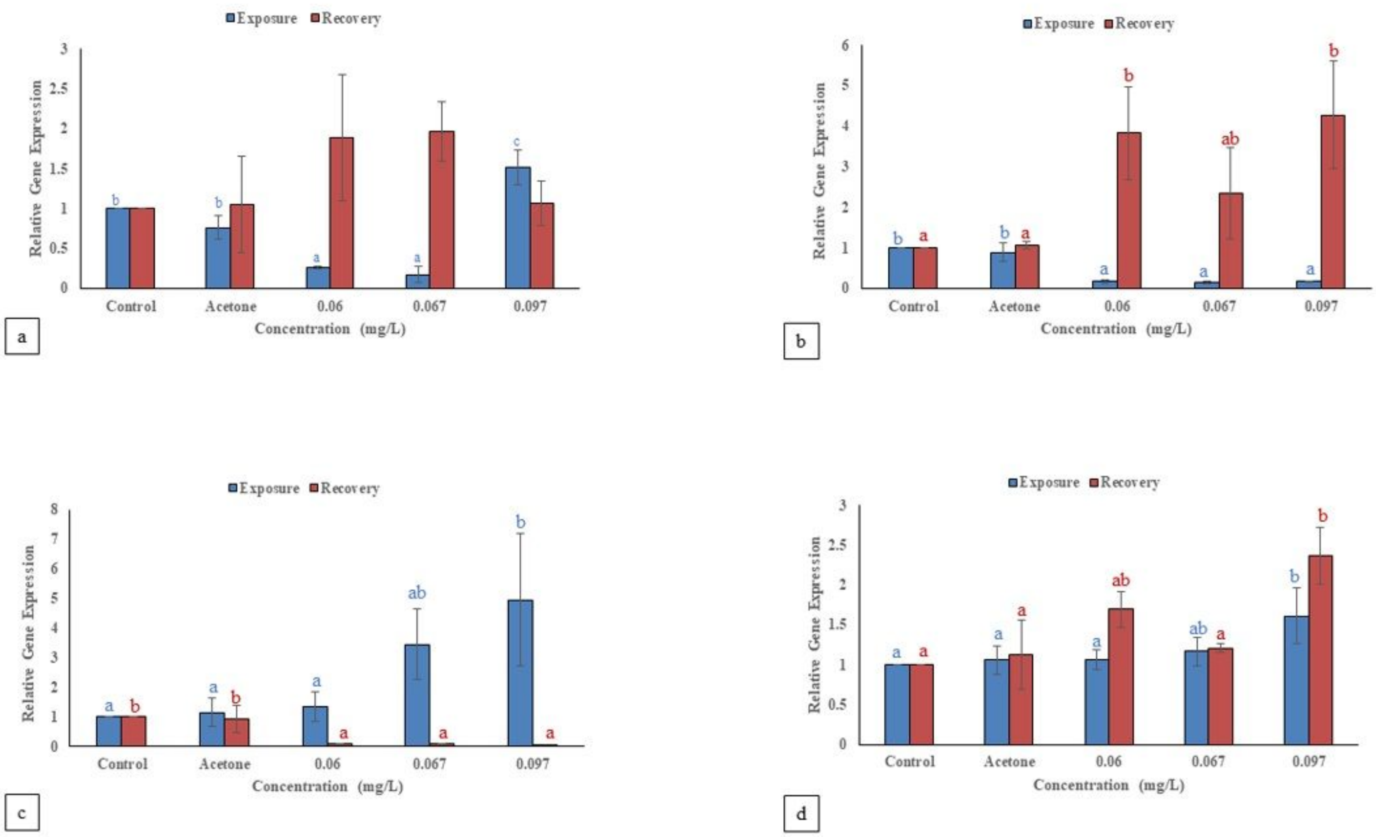

Figure 2

Effect of TCS on the expression of LDH (a), AST (b), ALT (c) and Ach (d) in the hatchlings of L. rohita. Values are mean ± SD, n=3 and different alphabets represent significant (P≤0.05) difference among treatments.

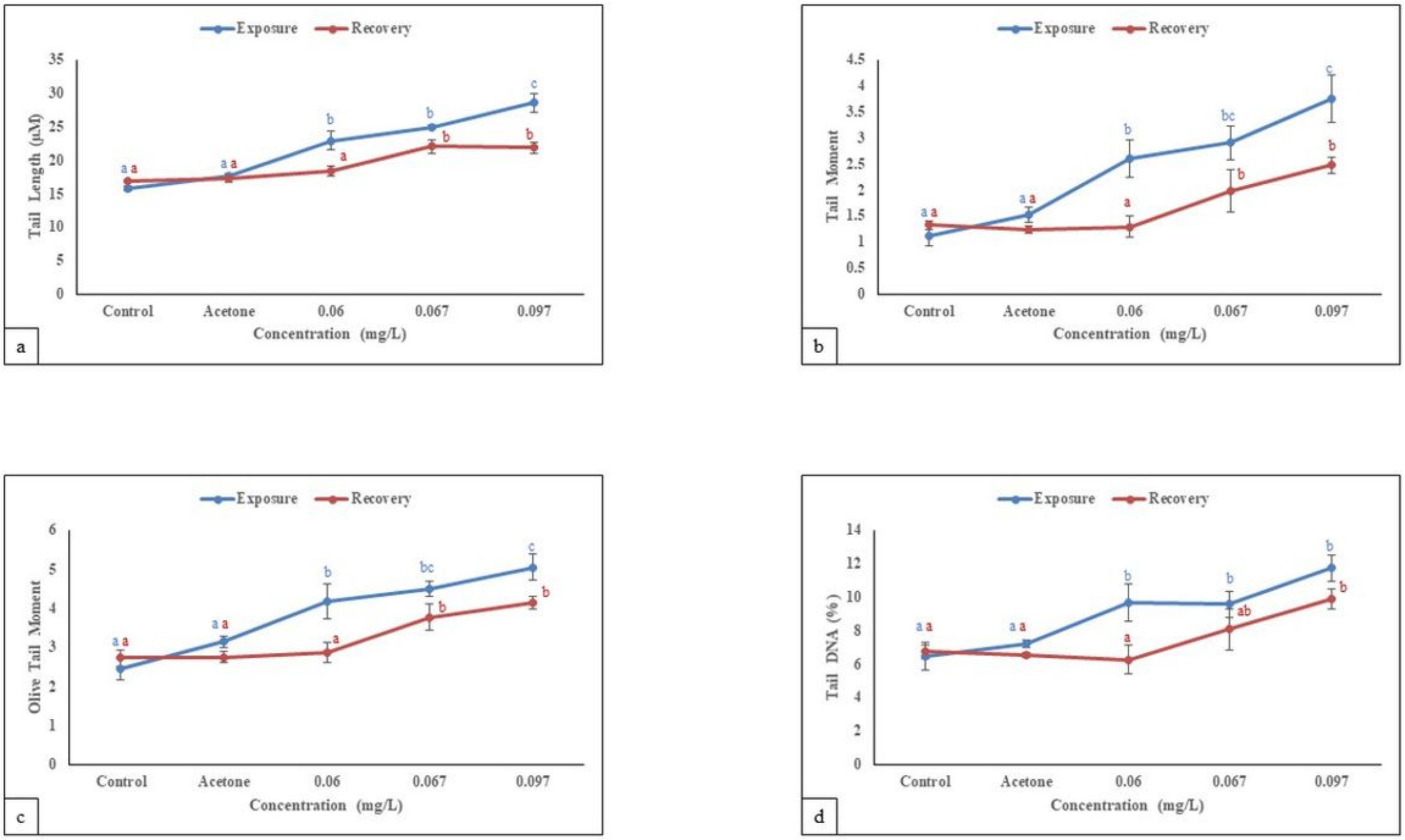

Figure 3

Variation in Tail length (a), Tail moment (b), Olive tail moment (c) and % tail DNA (d) in the hatchlings of L. rohita. Values are mean ± SD, n=3 and different alphabets represent significant (P≤0.05) difference among treatments.

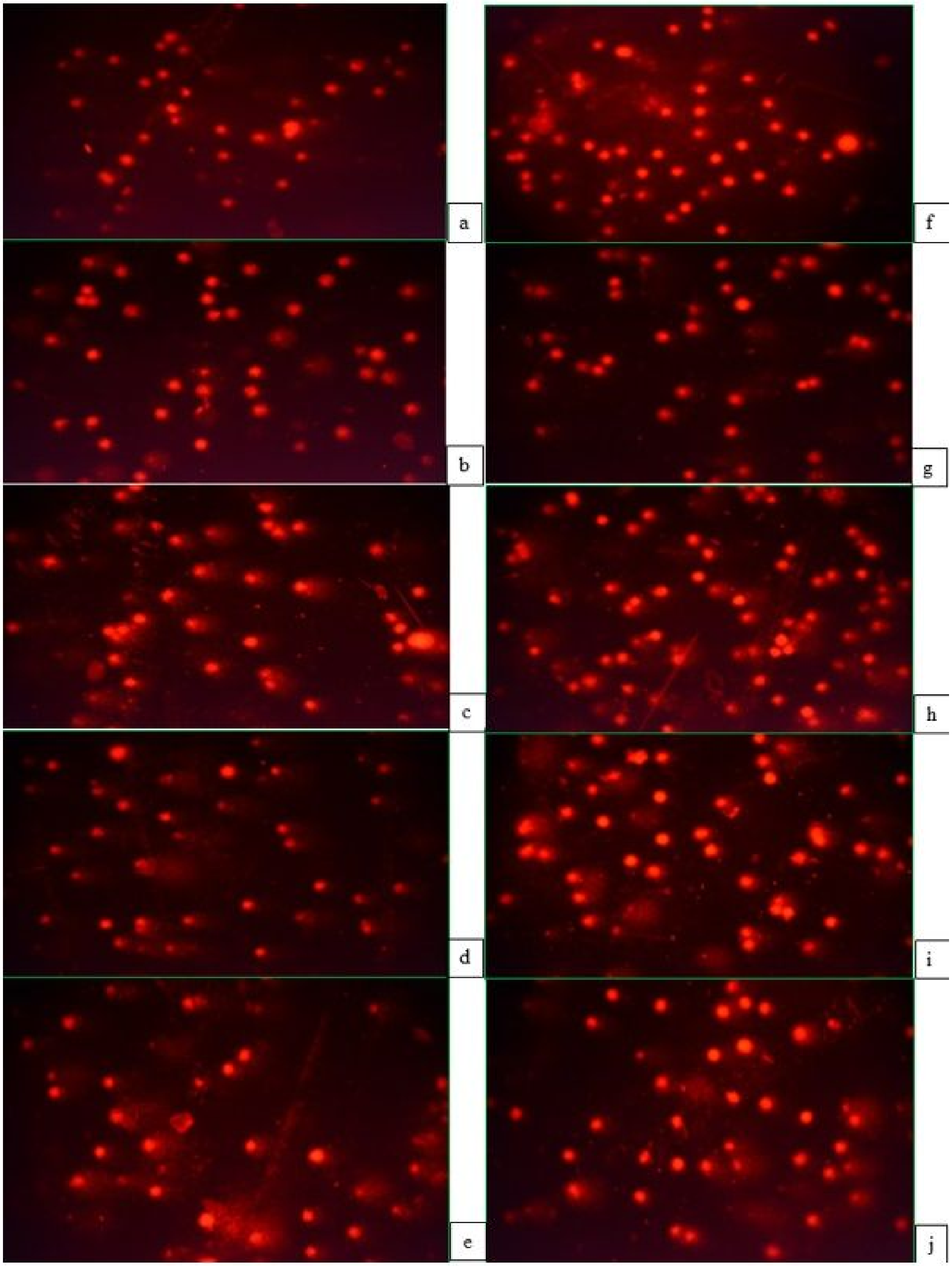

Figure 4

Photographs of comets after 96h exposure (a-e) and recovery period (f-j). Control (a, f), Solvent control (b, g), 0.06mg/L (c, h), 0.067mg/L (d, i) and 0.097mg/L (e, j).