Impact of perchlorate on a freshwater fish *Rasbora dandia*- hormonal, and biometric study

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Abstract

Perchlorate (ClO_4^-) is an emerging pollutant affecting aquatic ecosystem as well as human health and widely reporting from ground as well as surface waters near military sites and manufacturing units. In the present study we observed morphological changes on a fish (Rasbora dandia) collected from a natural pond contaminated with perchlorate. We conducted toxicity studies on laboratory condition by exposing healthy Rasbora dandia to ClO_4^- at concentration 6 to 14 mg/L. The LC 50 was calculated as 1970 mg/L as 96h. In the present investigation, we studied the $ClO4^-$ induced toxicity response of a fresh water fish (Rasbora dandia) under laboratory condition and revealed that prolonged exposure of environmentally relevant concentration of ClO_4^- can induce hormonal and biometric variations in this species. Hormonal studies showed that T_3 and T_4 concentration was significantly decreased at higher concentration and significant increase in TSH. The present study was limited to only one species and may extent up to other aquatic life of an ecosystem. To our knowledge this is the first report of perchlorate induced toxicity response of Rasbora dandia.

Key words

Chronic exposure, Biometric indices, Perchlorate, Rasbora dandia, Thyroid hormone

1. Introduction

Perchlorate (ClO_4^-) has been identified as a persistent pollutant which cause detrimental health effect and by acting as an endocrine disruptor (Wu *et al*., 2012). Perchlorate is a naturally occurring and manmade compound, whereas anthropogenic source are the main cause of contamination of aquatic ecosystem (Trumpolt *et al*., 2005; Morrison *et al*., 2006). The main mechanism of perchlorate toxicity is associated to the thyroid gland and thyroid hormone production.The fate and effect of perchlorate in an aquatic environment is of immense concern, because perchlorate salts are soluble, stable and persistent in water (Urbansky and Shock, 1999). Due to high stability and low reactivity, ClO_4^- transport readily in surface waters and through soils to groundwaters (Sparks, 1995). In an aqueous medium perchlorate anion readily dissociate from its various cation and remains stable for a long period under normal environmental conditions (Urbasky, 1998). Perchlorate present in ground water can be transported and might reside for decades (Flowers and Hunt, 2000; Morrison *et al*. 2006). Due to these properties aquatic organisms are most susceptible to perchlorate toxicity. The important health effect of perchlorate in humans and other ecological receptors are the inhibition of iodide uptake and subsequent thyroid hormone synthesis (Wolff, 1998; Clark, 2000).

Perchlorate contamination was reported from various food materials such as rice, fruits, leafy vegetables and breast milk. Presence of perchlorate was continuously reported from various water sources such drinking water, groundwater, surface water, sea water, snow and rainwater (Martinelango *et al.*, 2006; Ye *et al.*, 2013) from various countries such as USA, Japan, Canada, England, Korea, China and India (Dasgupta *et al*., 2005; Munster *et al*., 2009, Kannan *et al*., 2009; Isobe *et al*., 2013). The potential source of environmental contamination of perchlorate was mainly from its manufacturing, storage, testing, disposal sites and from military installations (USEPA, 2011).ClO_4^-, toxicity study was reported from animals such rats , deer mice, amphibians ,birds ,lizards , fishes and mammals (Mukhi and Patino., 2007 and Sharma and Patino., 2013).

Among fishes perchlorate toxicity study was mostly done in Zebra fish (*Danio rerio*) (Mukhi and patino., 2007; Schmidt *et al.*, 2012), Fathead minnows (*Pimephales promelas*) (Crane *et al.*, 2005); Eastern mosquito fish (*Gambusia holbrooki*) (Bradford et al., 2006), threespinestickle back (*Gasterosterus saculeatus)* (Bernhardt *et al.*, 2011; Furin *et al.*, 2015; Petersen *et al.*, 2016). These studies reported that perchlorate cause morphological deformities (Mukhi and patino., 2007; Bernhardt *et al.*, 2006 and Bernhardt *et al.*, 2011) reproductive abnormalities (Park *et al*., 2006; Bernhardt and Von Hippel., 2008) altered thyroid hormone, disruption of thyroid follicles (Schmidt *et al.*, 2012; Petersen *et al*., 2016). In fishes the route of perchlorate exposure is through gills, integuments and gastrointestinal tract (Theodoraki *et al*., 2006).

Due to the toxicological and potential effects of perchlorate, numerous states and agencies proposed enforceable standards and guidance levels for perchlorate. In 1998, perchlorate was added to the Contaminant Candidate List (CCL) for drinking water by USEPA (USEPA, 1998). In 2002 the USEPA published a reference dose (RfD) of perchlorate in drinking water level (DWEL) of approximately 1µg/L (USEPA, 2002). In 2005, USEPA established an oral reference dose (RfD) of perchlorate was 0.0007 milligrams per kilogram body weight per day and drinking water equivalent level (DWEL) of 24.5 µg/L (USEPA, 2008). The current health advisory level for ClO_4^- is set as 15µg/L based on the reference dose recommended by National Academy of Sciences (NAS) (USEPA, 2008). According to the report of World Health Organization (WHO) the established provisional maximum tolerable daily intake (PMTDI) of perchlorate is 0.01 mg/kg body weight (WHO, 2010).

Based on our previous report in Kerala the highest concentration of perchlorate was reported from Kulakkadu pond in Aluva near an Ammonium Perchlorate Experimental Plant (APEP) Based on this report the current study was mainly focused to evaluate the effect of environmentally relevant concentration of perchlorate induced toxicity study in a fresh water fish (*Rasbora dandia)* under laboratory conditions.

2 Materials and Methods

As a preliminary assessment of perchlorate induced toxicity response, we compared morphological variations in *Rasbora dandia* collected from the contaminated pond (test)

compared with fish from the unpolluted site (control). Based on this preliminary assement perchlorate induced toxicity studies were conducted in laboratory.

2.1 Experimental Fish collection and holding

The fish selected for toxicity study was *Rasbora dandia* (Valenciennes, 1844). *Rasbora dandia* was selected for the toxicity study since it is commonly found in the freshwater ecosystem, easy availability and dominant species present in the perchlorate contaminated pond (study site). Healthy and adult *Rasbora dandia* were collected from a local fish collector for toxicity studies. The fish were transported to the lab in live condition with care to avoid any mechanical injury. They were kept in a aquarium tank having 2000 L capacity filled with de chlorinated tap water and provided continues aeration. Prior to the experiment, fishes were acclimatized in laboratory conditions for a period of two weeks. During the acclimatization period fishes were fed with commercial fish food (Aqua mix pellet) twice per day. The physio -chemical parameters of the water were daily monitored using standard procedure (APHA, 2005) and maintained constant condition during the acclimatization period. The acclimatization was done at room temperature. Fishes free of any deformities, disease or lesions and showed good health conditions was selected for the experiments.

2.2 Chemicals and exposure

Pottassium perchlorate ($KCLO_4^-$) purchased from Sigma Aldrich was used for the prepration of stock solution. Test solutions were made by diluting the stock solution to produce desired perchlorate concentration for each treatment.

2.3 Chronic exposure study

Exposure study was carried in five rectangular glass aquaria having 500 L capacity filled with 300 L ClO_4^- solution. The aquaria were labeled from T1, T2, T3 T4 and T5 and filled with different concentration of ClO_4^- solution (6, 8, 10, 12 and 14 mg/L) respectively along with a control similar to the concentration (6 to 14 mg/L) reported from the perchlorate contaminated pond (study site) (Anupama *et al* 2018). Healthy adult fishes irrespective of sex, having uniform size were randomly divided into 6 groups (6.1 ± 2.4 cm of total length, 4.2±1.6 g of weight) and

introduced into each aquarium (30 fishes per aquarium). Fishes were fed with commercial food (Aqua mix pellet) daily during the experiment period. During the experimental period 75 % of the perchlorate solution was replaced with fresh solution twice a week for a period of 90 days. A control aquarium was also kept (500 L capacity) under the same condition without perchlorate. The experiment was conducted in triplicate for both (test and control). During the experiment at regular intervals (two weeks) water samples were collected from each aquarium for perchlorate and physico chemical analysis. The total period of exposure (duration of experiment) was 90 days. At the end of the experiment, fishes from each aquarium were collected. Blood samples and tissues from six groups of fish were carefully collected and used for hormonal studies.

2.4 Behavioural and morphological assay:

Behavioural monitoring of the experimental fish (both test and control) was done on daily basis throughout the exposure period. Behaviour changes such as fish general behaviour, feeding activity, swimming activity and opercular movement were monitored daily. For morphological analysis total length and wet body weight of the fish were measured and observed any deformities in scales, fins, gills and body also.

2.5 Biometric indices: After collecting blood fishes were scarified, liver and gonads were removed and weighed. biometric parameters such as hepato somatic index (H.S.I)and condition factor (K) were calculated by following the methods of Aysar *et al.,* 2005 respectively using the following equations,

Condition factor, $K = W \times 100 / L^3$ ..... (Eq. 1)

HSI = liver weights (g) / bodyweights (g) × 100 …….(Eq. 2)

2.6 Thyroid hormone analysis:

At the end of the experiment five fish from each tank were collected for hormonal analysis. Blood samples were collected from the fishes from the region of caudal peduncle by using a heparinised syringe which contain anticoagulant and kept in a vial for hormonal assay. From

each group (test and control) serum thyroxine (T_4), Triiodothyronine (T_3) and serum thyroid stimulating hormone (TSH) were analysed. Hormone analysis was done by using Chemiluminisentimunoassay (CLIA) method and following the manufacturer's standard procedure. The assays were done in triplicates and values are presented as mean ± standard deviation and represented in terms of µg/ml

Data analysis

The data obtained in the study were analyzed by using computerized programme, SPSS ver.19. Analysis of variance (ANOVA) was carried out to calculate the level of significance of variation between control and experimental group. Duncan multiple range test (DMRT) (Duncan, 1955) was used for the comparison of the data.

3. Result and Discussion

3.1. Toxicity response of *Rasbora dandia* collected from the contaminated pond

Fishes collected from the contaminated pond showed poor pigmentation compared to control fish. However there were no significant variation were observed in standard length, wet weight and condition factor of the fishes collected from the contaminated pond and control site. Hormone analysis showed that significant increase in TSH concentration (38.9%) was observed in fishes collected from the contaminated pond compared to control. However a slight decrease was observed among T_3 and T_4 concentration (Table 1).

Perchlorate is a thyrotoxicant and competitively inhibit iodide uptake into the thyroid through the sodium iodide symporter (NIS) (fisher *et al.*, 2000; Soldin *et al*., 2001).The decreased iodide uptake finally leads to the decreased thyroid hormone production thyroxine (T3) and triiodothyrronine (T4) and increased production of TSH through a feedback mechanism present in the hypothalamic –pitutary -thyroid axis (Siglin *et al*., 2000; Yu *et al*., 2002). Previous studies reported that perchlorate cause decline of T4 concentration in mosquito fish and Zebra fish (Bradford *et al*., 2006).

3.2 Behavioural and morphological changes on fish exposed to perchlorate

Behavioural and morphological changes are considered as a good biomarker to access the health status of fish (Kaushal *et al*., 2011). In the present study we observed morphological and behavioural changes of *Rasbora dandia* in response to perchlorate were showed in Table 2. The exposed fishes showed faster opercular activity (75-78 / m) than that of the control fish (62-65/m). Similarly exposed fishes showed reduced body coloration, invisible lateral line pigmentation and erratic swimming activity. In exposed group the prominent blue black stripes from the eyes to the base of the caudal fin of the fish is completely invisible during the exposure period. However such changes were not observed in control fish. These findings are similar to the previous reports of perchlorate induced toxicity study in fathead minnows *(Pimephales promelas*) and three spine stickle back (*Gasterosteus aculeatus*).) previous studies reported that perchlorate (10mg/L and 100mg/L) causes morphological abnormalities such as reduction of body pigmentation, poor scale formation, low wet weight, standard length and retarded growth in fathead minnows *(Pimephales promelas*) and in three spine stickle back (*Gasterosteus aculeatus*)(Crane *et al* ., 2005). Similarly chronic perchlorate exposure in three spine stickle back caused abnormal lateral plate development, slower growth rate, reduced body pigmentation and decreased swimming performance (Bernhardt and Von Hippe., 2008; Bernhardt *et al*., 2000,).These reports were also in consistent with the our observations.

3.3 Biometric Indices

Hepatosomatic index (HSI) is a general measurement to the overall condition of the fish and an appropriate biomarker for the substances which are toxic to liver (West., 1990). In the present study a significant difference were observed in the HSI of the treated fishes compared to control ($P \geq 0.05$). The HSI value was significantly increased from low concentration to high concentration and maximum HSI was reported from fishes exposed to 14 mg/L (Fig 1). The HSI value of the fishes was increased from 0.769 ± 0.102 (mean ±S.D) (control) to 1.244 ± 0.362 at low concentration (6mg/L) and 2.441 ± 0.386 at high concentration (14mg/L). The HSI value was maximum at highest concentration (14mg/L) (2.441 ± 0.386) and minimum at low concentration (6mg/L) (1.244 ± 0.362).Liver is the metabolic organ and the liver index is a useful biomarker to detect the harmful effect of the environmental stressors (Pait and Nelson.,

2003). Present study the high HSI is an indicative of liver enlargement due to perchlorate. From this experiment it was observed that prolonged exposure of perchlorate cause changes on the liver index of *Rasbora dandia*. Previous studies reported that acute perchlorate exposure caused fibrosis, steatosis and necrosis in hepatic tissue of *Poecilia sphenops* and liver glycogen depletion in Zebra fish (Schmdt *et a*l., 2012).

3.4. Condition Factor (K):

Present study showed that the total length, wet weight and condition factor of the treated fishes were not significantly different from the control (Table 3) ($p \leq 0.05$).The condition factor of the treated group were ranged from (mean ± S.D) 1.028 ± 0.047 (6mg/L) to 0.976 ± 0.031 (14 mg/L) compared to control (1.114 ± 0.209).There is no significant difference were observed at low concentration (6 to 12 mg/L) but only a slight difference were observed in fishes exposed to highest concentration(14mg/L). Similar result were also observed in previous study with zebra fish exposed to different concentration (11, 90, 131, 11480 mg/L) of perchlorate after 12 weeks exposure(Mukhi *et al*., 2005).

3.5 Thyroid hormone analysis

Present study showed that significant decrease were observed in concentration of serum T_3 and T_4 in ClO_4^- treated fishes, while significant increase were observed in TSH compared to control. The concentration of T_3 hormone reduced from (mean ± SD) 1.720 ± 0.012 µg/ml (control) to 0.970 ± 0.031 µg/ml at low concentration (6 mg/L) and 0.577 ± 0.015 µg/ml at high concentration (14mg/L) respectively (Fig.2). These result showed that in ClO_4^- exposed fishes T_3 concentration was reduced to 43.02% at lower concentration (6 mg/L) and 66.45% at highest concentration (14 mg/L) respectively compared to the hormone concentration of control fish. While the T_3 hormone level of the fishes exposed at 8 mg/L, 10mg/L and 12mg/L ClO_4^- concentration, were significantly reduced to 49.24%, 57.55% and 61.80% respectively compared to control. From this result it is observed that significant changes were observed in the T_3 hormone concentration of the ClO_4^- exposed fishes in respect to increasing ClO_4^- concentration.

Here the analysis of variance (ANOVA) showed significant ($P<0.01$) difference in T_3 hormone concentration in exposed fishes with respect to ClO_4^-concentration. Duncan's multiple range tests showed significant variation of T_3 hormone between ClO_4^-concentrations.

The concentration T_4 hormone reduced from (mean ± SD) 0.960 ± 0.010 µg/ml (control) to 0.533 ± 0.021 µg/ml at low concentration (6 mg/L) and 0.310 ± 0.010 µg/ml at high ClO_4^- concentration (14mg/L) respectively(Fig.2). These result showed that among ClO_4^- treated fishes the T_4 concentration was reduced to 44.47% at lower concentration (6 mg/L) and 67.71% at highest concentration (14mg/L) respectively compared to the hormone concentration of control fish. While the T_4 hormone concentration of the fishes exposed at 8 mg/L, 10mg/L and 12mg/L ClO_4^- were correspondingly reduced to 50.72%, 56.25% and 59.68% respectively compared to control. These result showed that the significant changes were observed in the T_4 hormone concentration of the ClO_4^- treated fishes with increasing ClO_4^- concentration. Here the analysis of variance (ANOVA) showed significant ($P<0.01$) difference in T_4 concentration in exposed fishes with respect to ClO_4^-concentration. Duncan's multiple range tests showed significant variation in T_4 hormone between ClO_4^- concentrations.

In the present study the concentration of TSH in ClO_4^- treated fishes was significantly increased compared to control group. The TSH hormone concentration (mean ± SD) was significantly increased in fishes exposed to highest concentration of ClO_4^- (14 mg/L) (0.633 ± 0.015 µg/ml) compared to control group (0.420 ± 0.010 µg/ml) (Fig.2). In this study the TSH level in fishes exposed to highest concentration (14 mg/L) of ClO_4^- was increased up to 33.6% compared to control. While at lower concentration there is no significant changes were observed. Here the analysis of variance (ANOVA) showed significant ($P<0.01$) difference in TSH concentration in exposed fishes with respect to ClO_4^-concentration. Duncan's multiple range tests showed that significant changes were observed in TSH hormone concentration in between control, 8mg/L, 10mg/L, 12mg/L and 14mg/L. However there is a non significant variation observed between control and 6 mg/L, 6 and 8 mg/L, 10 and 12 mg/L.

The result obtained from the present study revealed that environmentally relevant concentration of ClO_4^- can alter the thyroid hormone level in *Rasbora dandia* since thyroid hormone is a good

biomarker for ClO_4^- toxicity. Moreover the result of the present study emphasize that after 90 days of ClO_4^- exposure the T_4 hormone concentration is altered to a great extent. Similar result was reported from Zebra fish during 12 weeks of ClO_4^- exposure (Mukhi *et al*., 2005). In fishes and mammals the changes in T_4 level is not always correspond with the change in T_3 level (Blanton and specker, 2007). The best known biological impact of ClO_4^- in animal is the impairment of thyroid gland function. ClO_4^- altered thyroid function by inhibiting the iodine uptake into the sodium iodide symporter and prolonged exposure of ClO_4^- causes hyper stimulation of the thyroid gland (Soldin *et al.,* 2001).

Several previous studies reported that environmentally relevant concentration of ClO_4^- can altered thyroid hormone synthesis (Goleman *et al*., 2002). A study on Zebra fish reported that due to ClO_4^- exposure (100mg/L for 16weeks) T_4 hormone was reduced up to 95 % compared to control fish (Mukhi and Patino, 2007). Bradford *et al*. (2006) reported that 30 days ClO_4^- exposure decreased whole body T_4 concentration in Eastern Mosquito fish (*Gambusia holbrooki*). Similarly Crane *et al.* (2005) reported that due to perchlorate exposure (100mg/L) the production of T_4 content decreased in Fathead minnows, *Pimephales promelas*. Previous studies reported that ClO_4^- caused changes in thyroid hormone concentration in other organisms such as Sea lamprey, rabbits and African clawed frog (York *et al*., 2005; Goleman *et al*., 2002). Manzon and Youson (1997) observed that ClO_4^- caused decline of serum T_4 and T_3 concentration in sea lamprey *(Petromyzon marinus)*. Goleman *et al.* (2002) reported that after 70 days of ClO_4^- (14140 ppb) exposure leads the fall of T4 concentration in *Xenopus laveis*. York *et al*. (2001) suggested that oral administration of ammonium perchlorate (≥ 30 mg/kg/day) caused decreased production of T_4 concentration in rabbit. From the present study it was observed that at environmentally relevant concentration of ClO_4^- can impair thyroid hormone synthesis and cause significant changes in thyroid hormone concentration in *Rasbora dandia*. This data is consistent with the previous report of Brechner *et al*. (2000) and Goleman *et al*. (2002). Disruption of the thyroid homeostasis may affect growth, development and reproduction in fishes and this effect may finally leads from the population to ecosystem levels (Power *et al*., 2001).

Conclusion

In conclusion present study showed that environmentally relevant concentration of ClO_4^- can altered thyroid hormone production in *Rasbora dandia*. The altered thyroid hormone production observed in *Rasbora dandia* may indicate a potential health risk of the species and may extent to other fishes present in the ClO_4^- contaminated pond. Present study therefore highlights perchlorate contamination present in a natural ecosystem is harmful to the fish population and which may be adversely affected on the in natural ecosystem. This study is useful for assessing the possible environmental risk of a fresh water fish present in a perchlorate contaminated ecosystem. Perchlorate contamination in an aquatic ecosystem will be highly toxic to organisms especially fishes and can extend up to human beings. Therefore pollution prevention measures must be taken without delay to prevent harmful effects of ClO_4^- in aquatic biota of this pond.

In conclusion, the present study underlines the need for a close monitoring of ClO_4^- contamination in Kerala and also for implementing regulatory measures for controlling the environmental release of ClO_4^- in Kerala or in India as a whole. Present study highlight the need for more rigorous control of the endocrine disruptor present in a natural ecosystem and thereby ensuring protection of aquatic as well as human health. Indiscriminate discharge of such type of pollutant must lead to ecological risk and destruction of a natural ecosystem. So government policy makers should take effective management strategies to control such type of persistent pollutant found on the natural ecosystem.

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Table .1 The level of T_4, T_3 and TSH of fishes collected from the contaminated pond and control site.

Hormones	Pond site	Control site
T4(ug/ml)	0.72 ± 0.06	0.76 ±0.38
T3 (Ug/ml)	0.98 ± 0.14	0.93 ± 0.62
TSH (Ug/ml)	0.54 ± 0.21	0.33 ± 0.98

Table 2. Behavioural and morphological changes observed in *Rasbora dandia* exposed to perchlorate.

Behavioural changes.	**Experimental group.**	**Control group.**
Opercular movement	Increased (75-78/m)	normal(62-65/m)
Swimming activity	Erratic	normal
Dorsal and ventral fin	sometimes erected	not erected
Feeding response	active	active
Morphological changes		
Body pigmentation	reduced	normal
Coloration of fins	decreased	normal
Lateral line pigmentation	completely invisible	visible

Table 3. Effect of perchlorate on total length, body wet weight and condition factor of *Rasbora dandia* after 90 days exposure

Concentration (mg/L)	Total Length (mean ±SD)	Body Wet Weight (mean ±SD)	Condition Factor (mean ±SD)
0	8.040 ± 0.114	5.320 ± 0.179	1.114 ± 0.209
6	8.020 ± 0.239	5.300 ± 0.265	1.028 ± 0.047
8	8.060 ± 0.305	5.240 ± 0.321	1.002 ± 0.053
10	7.980 ± 0.349	5.200 ± 0.255	1.028 ± 0.087
12	7.960 ± 0.297	5.220 ± 0.228	1.039 ± 0.087
14	8.160 ± 0.207	5.300 ± 0.265	0.976 ± 0.031

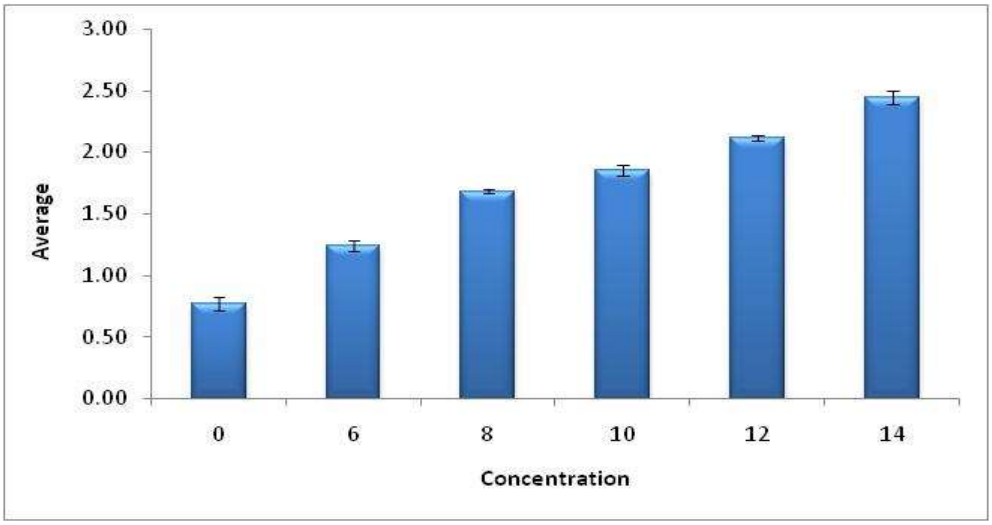

Fig 1. Effect of perchlorate on Hepatosomatic Index (HSI) of *Rasbora dandia*

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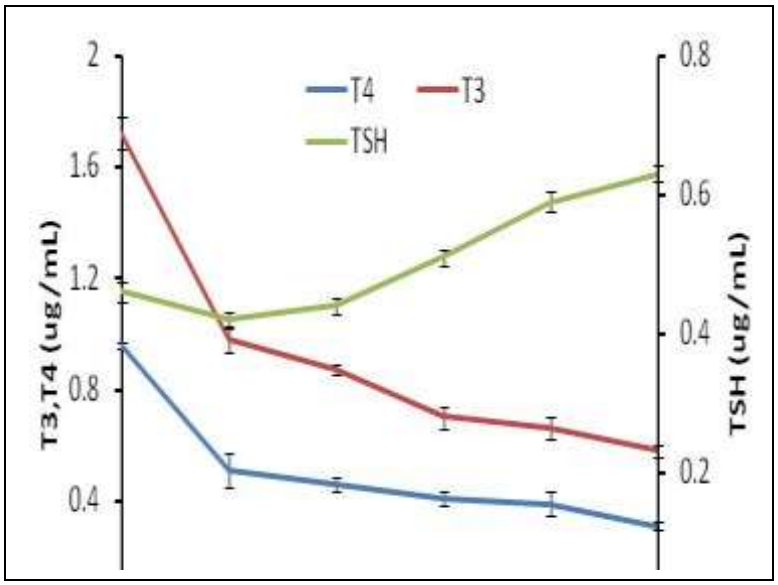

Fig.2. Total T3, T_4 and TSH concentrations in plasma of *Rasbora dandia* exposed to perchlorate at 90 days

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