STUDIES ON HORMONAL CHANGES IN THE THYROID OF A FRESHWATER TELEOST, CHANNA STRIATUS (BLOCH) ON EXPOSURE TO ENDOCRINE DISRUPTOR CHEMICAL, TRICLOSAN

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ABSTRACT
Triclosan [5 chloro 2 (2, 4 dichlorophenoxy)] phenol is a broad spectrum antibacterial agent commonly used in personal care, veterinary, industrial and household products. Presently it has been detected in aquatic ecosystems. Thyroid hormones play a pivotal role in somatic growth, development, and metamorphosis of vertebrates. The structural similarity of thyroid hormones versus Triclosan shows that Triclosan can have adverse effects on the thyroid system. To date, most of the studies have focused on estrogenic and androgenic endocrine disruptors, but reports on Thyroid endocrine disruptors are comparatively less. In the present study, Channa striatus was utilized as a model organism to assess the effect of endocrine disruptor chemical Triclosan on Thyroid hormones for 28 days. The LC$_{50}$ value for 96 hours was found to be 0.602ppm. 1/10th of the LC$_{50}$ concentration (0.0602 ppm) and 1/5th of the LC$_{50}$ concentration (0.120ppm) of the Triclosan was taken as sublethal concentrations. The Triclosan was found to significantly decrease (p<0.05) the T3 and T4 levels in the serum of the fishes, whereas TSH level significantly increased (p<0.05). The alterations of the hormonal levels and Thyroid gland may be used as a potential biomarker and can also establish the endocrine disrupting property of Triclosan in vertebrates.

Keywords: Triclosan, Channa striatus, Thyroid hormones, LC$_{50}$.

INTRODUCTION
The teeming modern application of chemicals in an extensive variety of commodities makes a constant susceptibility of humans and wildlife to chemicals with the potential of conflicting with biological systems. Various chemicals are speculated to be fatal to human well-being, extending from effects on the immune system, nervous system, and reproductive system. Some environmental chemicals are notably hypothesized to have thyroid-disrupting properties. The mechanisms included in the endocrine disruptor negotiated a modification of the thyroid function has been widely studied but are still not be fully recognized. Endocrine disrupting chemicals intervene with hormone synthesis in the thyroid gland (Ishihara et al., 2003; Brown et al., 2004; Boas et al., 2006). They competitively bound to thyroid hormone binding proteins in blood alike transthyretin (TTR) (Wade et al., 2002), to the membrane-bound transporters of target cells (Gauger et al., 2004) or to intracellular cytosolic thyroid hormone binding proteins which are believed to function as modulators of nuclear-receptor-mediated transcription (Blanton and Specker, 2007). The endocrine disruptors can similarly perform on metabolic enzymes which activate or inactivate thyroid hormones (Zoeller et al., 2007). Peripheral iodothyronine deiodinases regulate the metabolism of thyroid hormones in different organs and are therefore indispensable in the management of levels of biologically active T$_3$ (Boas et al., 2006). Eventually, pollutants can disrupt thyroid hormone receptors and accessory proteins which immediately regulate the gene expression in thyroid hormone responsive elements (Blanton and Specker, 2007). Besides the immediate effects through these sites, auxiliary results through the hypothalamus and anterior pituitary gland are also plausible (Zoeller et al., 2007). Endocrine disruption of thyroid function may possess critical risks to thyroid hormones to act the significant role in the physiological condition in vertebrates. Thyroid hormones aid in charge of various physiological
functions according to the growth of the embryo, larva, juvenile and mature fish (Janz, 2000). The thyroid hormones play critical regulative functions in several essential features of the life records of vertebrate animals, including organogenesis, reproductive biology, metabolic control, and, thermogenesis in poikilotherms. In mature fish, thyroid hormones show fundamental significance in the control of before-mentioned primary physiological means as growth, nutrient utilization, and reproduction. Fish breed quicker and are stronger when the thyroid hormone levels are raised (Power et al., 2001; Yamano, 2005), which denote a commercial break in fishery including aquaculture. Thyroid hormones comprise primarily of iodinated thyronine compounds, with iodide remaining attached to the 3’ and 5’ positions of the phenolic rings. The three principal forms of iodinated tyrosine’s in the blood of vertebrates comprise the tetra-iodothyronine (3, 32, 5:52 - thyroxine, T4), the tri-iodothyronines (3,32,5-I triiodothyronine, T3), and 3, 32, 52 - triiodothyronine (reverse T3, rT3). Among those, T3 is the biologically potent form and its hormonal impact by binding to particular T3 receptors (TRs) that are associated with chromatin in the nucleus of the specific cells, and by so achieving, controlling the expression of specific genes. Some of the iodinated thyronines, including diiodothyronine, may also perform at extranuclear sites (e.g., the mitochondria or cell membranes) (Lanni et al., 1992). Thyroid disrupting chemicals (TDCs) are broadly described as xenobiotics that disrupt thyroid hormone (TH) signaling. The neurological development of mammals is considerably reliant on TH homeostasis, and it is susceptible to the disruption of the thyroid axis. A wide range of structurally diverse TDCs hinders with the hypothalamic–pituitary–thyroid (HPT) axis by various mechanisms to change TH homeostasis, at the receptor level, in binding to transport proteins, in cellular uptake mechanisms, or in altering the metabolism of THs (Boas et al., 2006). TH homeostasis requires a complicated interaction of homeostatic regulative processes. Regulation of THs involves control of iodine uptake, synthesis and storage of THs in the thyroid gland, deliver into and carrier of THs within or out of circulation, tissue-specific deiodination, and degeneration by hepatic catabolic enzymes, and so on. Interest on TDCs has risen because of the significant role that THs do in brain development (Crofton, 2005). Despite the temporary disruption of normal thyroid homeostasis will direct to harmful effects, particularly in the developing nervous system (Crofton, 2008). The modifications of conventional TH levels can skeptically influence pregnancy issue, fecundity, and postnatal growth in humans and animals. Particularly, when mutations happen in a crucial developmental stage, such as lack of TH during pre- and early postnatal period, it may generate seriously and resolute damage to the infant, following in irregular brain development identified as cretinism in individuals, screening for neonatal hypothyroidism is actively accompanied in most industrialized nations to halt infrequent cretinism caused by inherent aberrations (Kolbuchi, 2009). The chemically alike THs in the vertebrates are similar in amphibians and fishes, where they perform functions distinctly and significantly. Triclosan has been listed out to disrupt TH signaling and lower serum T4 in male juvenile rats (Zorrilla et al., 2009). Triclosan upregulated phase II glucuronidation and sulfation, and this enhanced catabolism of T4 may be somewhat liable for the triclosan-induced hypothyroxinaemia negatively causing TH homeostasis (Paul et al., 2010). Antagonistic effects of Triclosan on thyroid hormone (TH) homeostasis have been recorded in rats and frogs. Short-term oral Triclosan exposure ended in hypothyroxinaemia on weanling rats (Crofton et al., 2008). Stoker and colleagues described that Triclosan reduced T4 without significantly altering thyroid-stimulating hormone (TSH) following 31 days of oral Triclosan exposure (Zorrilla et al., 2008). The interdisciplinary studies show that the environmental susceptibility to industrial chemicals may impose a serious menace to human and wildlife thyroid homeostasis (Boas et al., 2009). Thus, thyroid toxicants are of huge interest in the scientific field.

MATERIALS METHODS

Experimental Organism

*Channa striatus* used in this study was collected locally and was acclimatized to laboratory conditions for 14 days before the experiment phase. Healthy fishes, weighing 42±4.8g and with a length of 13±4.5cm were transferred from holding tanks to experimental aquariums. Fishes were fed with feed prepared in the laboratory and feeding was stopped during the experimental period. To study the effect of Triclosan on the Thyroid gland and its hormones, fishes were exposed to 1/5th (0.120ppm) and 1/10th (0.0602ppm) concentration of 96 hour LC50 (0.602ppm) of Triclosan for 7, 14, 21 and 28 days respectively. After each exposure period, blood and thyroid tissue from live fishes were collected with proper care and used for analysis. The blood was collected in small vials by puncturing caudal peduncle and heart. 5ml of blood was collected from caudal peduncle and heart of the fishes. Following collection, the blood was let to clot by leaving it undisturbed at room temperature for 15 minutes. The clot was removed by centrifuging at 2,000 x g for 10 minutes in a centrifuge. The resulting supernatant was collected as serum.
Hormonal analysis
The concentrations of various hormones such as thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) were estimated in the serum of control, and sub-lethal Triclosan treated *Channa striatus* for different periods such as seven days, fourteen days, twenty one days and twenty-eight days. The selected hormones were estimated in triplicate based on the principle of Elecsys ECLIA assay.

Analysis of serum Thyroid hormones
Serum TSH, T4, and T3 were estimated by employing Electro Chemiluminescence Immuno Assay (ECLIA) using Elecsys 2010 Roche. Serum levels of hormones of control and sub-lethal Concentrations of Triclosan exposed *Channa striatus* were expressed as μIU/mL for thyroid stimulating hormone TSH, ng/dL for triiodothyronine (T3) and μg/dL for thyroxine (T4).

Analysis of serum TSH
50 μL of sample, a biotinylated monoclonal TSH specific antibody and a monoclonal TSH-specific antibody labeled with a ruthenium complex react to make a sandwich complex. Following the addition of streptavidin-coated microparticles, the complex converts to the solid phase through the interaction of biotin and streptavidin. Meanwhile, the reaction mixture was aspirated into the measuring cell where the microparticles are magnetically captured on the surface of the corresponding electrode. Unbound substances are later separated by a photomultiplier. Outcomes are arranged through a calibration curve, which is instrument-specifically created by 2-point calibration and a master curve produced via the reagent barcode.

Analysis of serum T3
15 μL of the sample and anti-T3-specific antibody marked with a ruthenium complex. After addition of biotinylated T4 and streptavidin-coated microparticles, the binding sites of the labeled antibody become filled, by the formation of an antibody-hapten complex. The whole complex was associated with the solid phase through the interaction of biotin and streptavidin. The reaction mixture was aspirated in the measuring cell wherever the microparticles are magnetically captured upon the surface of the electrode. Unbound substances are then separated with Pro Cell. Administration of a voltage to the electrode later generates chemiluminescent emission which is estimated by a photomultiplier. Results are measured through a calibration curve which is instrument-specifically generated by point calibration and a master curve produced through the reagent barcode.

Analysis of serum T4
15 μL of the sample and a T4-specific antibody marked with a ruthenium complex. Following the addition of biotinylated T4 and streptavidin-coated microparticles, the free binding sites of the labeled antibody become conquered, with the formation of an antibody-hapten complex. The whole complex converted to the solid phase through the interaction of biotin and streptavidin. The reaction mixture was aspirated in the measuring cell wherever the microparticles are magnetically captured on the surface of the electrode. Unbound substances are then separated with Pro Cell. Applying voltage to the electrode then produces chemiluminescent emission which is regulated by a photomultiplier. Results are defined through a calibration curve which is instrument-specifically made by 2-point calibration and a master curve produced via the reagent barcode.

Statistical Analysis
All replicates (n=3) were examined for the calculation of mean values. Statistical analysis was done with SPSS Version 20.0 statistical software package. Differences in hormonal parameters between exposure times and concentration of dosage have been constrained to statistical analysis using Student’s t-test to examine the variations in comparison with control. The hormonal parameters were expressed as the mean ± standard error.

RESULTS AND DISCUSSION
The chemical coordination by the endocrine system in animals is distinguished to control many hormone-dependent physiological functions. This endocrine coordinating system is a possible victim of xenobiotics, and its vulnerability continues in part in the finely harmonized mechanisms by which the endocrine control system works in animals (Hontela, 1997). The xenobiotics that intrude in the environment can have either direct antagonistic influences on the endocrine gland and tissues or their effects can be long through changes of homeostasis and actions of non-endocrine organs (Atterwill and Flack, 1992).

The calculated levels of serum T₄ hormones in control and sublethal Triclosan treated *Channa striatus* is given in Table 1 and the same is shown in Fig 1.

The serum levels of T₄ in control *Channa striatus* ranged significantly from 6.30±0.14 g/dl to 6.77±0.03 g/dl. Whereas triclosan treated *Channa striatus* to sub-lethal concentrations of Triclosan showed significant reductions throughout the experiment period in serum T₄ levels. Sublethal Triclosan exposure caused a highly significant...
decrease in serum T4 levels to about 15.3%, 23.1%, 31.08% and 44.4% in 1/5th sublethal exposure and 9.3%, 11.1%, 14.8% and 21.1% in 1/10th sublethal exposure following 7, 14, 21, and 28 days respectively. T4 present in higher quantities than T3 in the blood circulation. Triclosan was discovered to repress thyroxine absorption through the pregrenne-X receptor, accordingly disrupting proper endocrine functions that should be driven out by this enzyme. Inhibition is caused by the resemblance in the shape and size of the Triclosan and thyroxine molecules (Hinther et al., 2011). Thyroxine, is the main hormone secreted by the thyroid gland, is responsible for regulating metabolism.

Triclosan chemically mimics thyroxine and binds to its receptor sites. Once blocked by Triclosan, the thyroid hormone cannot function. Dose-dependent decreases in total T4 were observed. Among the thyroid hormones, the circulating hormone in the blood is predominantly thyroxine (T4), but triiodothyronine (T3) is metabolically active, and the presence of T3 in a lesser amount (T4: T3 = 7:1) show a fast conversion from T4 to T3 before usage (Eales, 1979; Oppenheimer, 1979). Hazard evaluation studies on Triclosan shows that it is lethal for aquatic organisms, and results involve restricted or reduced embryonic development and hatching, altered enzyme activities, genotoxicity, and mortality in fish embryos and changes in swimming behavior and survival of adult fish (Oliveira et al., 2009). However, the methods of action of Triclosan in humans and wildlife are yet to be examined. Weak estrogenic or androgenic activities for Triclosan were reported from fish to mammals (Cahoreau et al., 2015). However, the structural identity with THs imply it may also be unfavorable impacts on the thyroid (Cullinan, et al., 2012).

Nevertheless, few understood mechanisms by which Triclosan interrupted the thyroid axis, exclusively remarkable current investigations showed a decrement in circulating Thyroid hormones. The discerned hyperplasia of the thyroid in Triclosan treated fish, which had more significant numbers of thyroid follicles compared to control fish, may remain as a response to the TSH stimulation. In the present study, a significant increment in TSH in Triclosan treated fish was an obvious decrease in the activity of thyroid hormone which is indicative of immediate action for Triclosan on the thyroid gland to disrupt Thyroid hormone synthesis and release.

In the present study, T4 and T3 levels in serum were decreased and are in line with earlier studies in fishes. The insensitivity of TSH is concerns as an important marker of the HPT axis and TH imbalance, and our conclusions are per other investigations on endocrine disrupting chemicals. The serum levels of T3 in control Channa striatus ranged from 2.17±0.02 ng/ml to 2.36±0.03 ng/ml. The sublethal Triclosan treated fish recorded a significant decrease in T3 throughout the study period. Sublethal Triclosan exposure caused a highly significant decrease in serum T3 levels to about 18.8%, 28.07%, 29.9% and 43.2% in 1/5th sublethal exposure 6.4%, 14.9%, 20.27% and 31.3% in 1/10th sublethal exposure following 7, 14, 21, and 28 days respectively (Table 2, Fig 2).

Thyroid stimulating hormone (TSH) produced by adenohypophysis is recognized to control iodine metabolism through its effect on the thyroid gland thereby stimulating thyroxine production (Hoar, 1975). TSH is known as a glycoprotein of the α-β dimer structure. TSH joins plasma membrane receptors of thyroid follicle cells and stimulates adenylyl cyclase. The sequential increment of cAMP (the second messenger) is liable for the effect of TSH in thyroid hormone biosynthesis. TSH possessed different acute influences on thyroid function (Cahoreau et al., 2015). Results of the serum levels of thyroid stimulating hormone (TSH) in control and sub-lethal Triclosan treated, Channa striatus are reported in Table 4 and depicted in Fig. 4. The serum levels of TSH in control Channa striatus ranged from 0.34±0.02mlu/ml to 0.36±0.01mlu/ml during different experimental periods. Sublethal triclosan exposure caused a highly significant increase in serum TSH levels to about 30.09%, 47.37%, 58.77% and 74.34% in 1/5th sublethal exposure and 10.62%, 19.3%, 28.07% and 36.28% 1/10th sublethal exposure following 7, 14, 21, and 28 days respectively. From our study, it was noted that Triclosan does not block TSH activity. A significant increase in TSH was observed at all exposure periods. TH exerted negative feedback on the expression of TSH. The reduction of thyroid hormones to a certain level, the pituitary secreted more TSH in the blood, and this could be the reason for the increase in TSH activity in the serum of the species under study. We observed an increment in TSH concentration while the T3 and T4 concentrations went down (Fig.3). The thyroid systems of fish and mammals are related in many regards, with one major exception. The mammalian system is operated essentially through the central brain-pituitary-thyroid axis that controls thyroid secretion of both T4 and T3. Instead of the thyroid system does not appear to be induced essentially by the central brain-pituitary-thyroid axis in fish (Leatherland, 1988). Alternatively, the primary brain-pituitary-thyroid axis in fish possesses the principal function of assuring T4 homeostasis. T3 generation and homeostasis are controlled in peripheral
tissues by the transformation of T4 to T3 by deiodination (Hoermann, et al., 2015).

a=sig difference b/w control & 1/5\textsuperscript{th} LC\textsubscript{50} Exposure,  
b=sig difference b/w control & 1/10\textsuperscript{th} LC\textsubscript{50} Exposure,  
c=sig difference b/w 1/5\textsuperscript{th} and 1/10\textsuperscript{th} LC\textsubscript{50} Exposure.

Means marked with alphabets a,b,c in the graphs are significantly different as calculated using the paired t-test at the 5% significance level.

Nevertheless, the examination of the thyroid alterations and the evaluation of the effect is further complicated because it is challenging to differentiate between immediate and incidental xenobiotic effects on the thyroid cascade, which possesses a substantial potential to interfere with thyroid hormones homeostasis (Zoeller and Tan, 2007). From our present study, it was observed that Serum T4 and T3 concentrations significantly decreased (p<0.05) while serum TSH concentration increased (p<0.05) in Triclosan treated fish as compared to the control. In the present study reduced T4 and T3 and raised TSH levels typically recommend hypo-functioning of the thyroid gland, which is a notable conclusion of our present study. Circulating T4 and T3 levels seemed as valuable biomarkers of the possible result on thyroid gland as an outcome of susceptibility to endocrine disrupting chemicals (Shumacher et al., 1993; De Guise et al., 1995). Serum levels of T4 and T3 serve as reliable indicators of the thyroid function in both human and laboratory animals. Any difference in their levels indicates interference in the glandular synthesis and secretion,

<table>
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<th>Parameters</th>
<th>Days</th>
<th>Control</th>
<th>1/5\textsuperscript{th} Concentration</th>
<th>1/10\textsuperscript{th} Concentration</th>
<th>% decrease in 1/5\textsuperscript{th} Concentration</th>
<th>% decrease in 1/10\textsuperscript{th} Concentration</th>
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<tr>
<td>T4 µg/dL</td>
<td>7 days</td>
<td>6.77±0.03</td>
<td>5.73±0.03</td>
<td>6.14±0.08</td>
<td>15.3%</td>
<td>9.3%</td>
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<td></td>
<td>14 days</td>
<td>6.66±0.03</td>
<td>5.12±0.03</td>
<td>5.9±0.06</td>
<td>23.1%</td>
<td>11.1%</td>
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<td>21 days</td>
<td>6.53±0.08</td>
<td>4.50±0.11</td>
<td>5.56±0.08</td>
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<td>14.8%</td>
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<td></td>
<td>28 days</td>
<td>6.30±0.14</td>
<td>3.50±0.15</td>
<td>4.97±0.08</td>
<td>44.4%</td>
<td>21.1%</td>
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<th>% decrease in 1/5\textsuperscript{th} Concentration</th>
<th>% decrease in 1/10\textsuperscript{th} Concentration</th>
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<tr>
<td>T3 ng/ml</td>
<td>7 days</td>
<td>2.34±0.10</td>
<td>1.90±0.06</td>
<td>2.19±0.08</td>
<td>18.8%</td>
<td>6.4%</td>
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<td>14 days</td>
<td>2.28±0.16</td>
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<td>1.94±0.11</td>
<td>28.07%</td>
<td>14.9%</td>
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<td>21 days</td>
<td>2.17±0.02</td>
<td>1.52±0.01</td>
<td>1.73±0.02</td>
<td>29.9%</td>
<td>20.27%</td>
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<td></td>
<td>28 days</td>
<td>2.36±0.03</td>
<td>1.34±0.01</td>
<td>1.62±0.01</td>
<td>43.2%</td>
<td>31.3%</td>
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<th>Control</th>
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<th>1/10\textsuperscript{th} Concentration</th>
<th>% Increase in 1/5\textsuperscript{th} Concentration</th>
<th>% Increase in 1/10\textsuperscript{th} Concentration</th>
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<tr>
<td>TSH (IU/mL)</td>
<td>7 days</td>
<td>1.13±0.02</td>
<td>1.47±0.086</td>
<td>1.25±0.028</td>
<td>30.09%</td>
<td>10.62%</td>
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<td>14 days</td>
<td>1.14±0.017</td>
<td>1.68±0.017</td>
<td>1.36±0.030</td>
<td>47.37%</td>
<td>19.3%</td>
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<td>21 days</td>
<td>1.14±0.020</td>
<td>1.81±0.017</td>
<td>1.46±0.02</td>
<td>58.77%</td>
<td>28.07%</td>
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<td>28 days</td>
<td>1.13±0.01</td>
<td>1.97±0.11</td>
<td>1.54±0.02</td>
<td>74.34%</td>
<td>36.28%</td>
</tr>
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Mean ± SE, n=3 Significant level p<0.05
SUMMARY AND CONCLUSION

Severe hormonal changes were noticed in the experiment fish on exposure to sublethal concentrations of Triclosan at various exposure periods. T4 - Thyroxine, the major hormone secreted by the thyroid gland, is responsible for regulating metabolism. Triclosan chemically mimics thyroxine and binds to its receptor sites. Once blocked by Triclosan, the thyroid hormone cannot function. Dose-dependent decreases in total T4 were observed. A maximum decrease of T4 was (44.4 %) in 1/5th sublethal exposure on the 28th day, and the minimum decrease was (9.3%) in 1/10th sublethal exposure on the 7th day. Reduction of the serum T3 levels is mainly due to a drop in T4 production and secretion. The reduction of thyroid hormone (both T3 and T4 in the present study), could be due to the competitive binding of Triclosan with the thyroxine (thereby making it inactive) thereby reducing the hormone activity. A maximum decrease of T3 was (43.2 %) in 1/5th sublethal exposure on the 28th day, and the minimum decrease was (6.4%) in 1/10th sublethal exposure on the 7th day. A maximum increase of TSH was (74.4 %) in 1/5th sublethal exposure on the 28th day, and the minimum increase was (10.62%) in 1/10th sublethal exposure on the 7th day. TH exerts negative feedback on the expression of TSH. The reduction of thyroid hormones to a certain level, the pituitary secretes more TSH in the blood, and this could be the reason for the increase in TSH activity in the serum of the species under study.

The comprehensive study recommends that the thyroid is sensitive to Triclosan toxicity and it is appropriate to examine the effect on thyroid gland using different exposure concentrations.
concentrations of Triclosan. There is also necessary to manage large-scale epidemiological investigations on humans concerning the effect of Triclosan. Whether exposure to Triclosan leads to thyroid related deformities in humans continues to be discovered. A significant ambiguity that impedes the risk evaluation of Thyroid disrupting chemicals is the absence of accurate characterization of the dose-response relationship between the degree of change in hormone concentrations and its adverse effects.

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