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Chronic Effects of Diazinon® Exposures Using Integrated Biomarker Responses in Freshwater Walking Catfish, *Clarias batrachus*

Shubhajit Saha [1], Azubuike V. Chukwuka [2], Dip Mukherjee [3], Lipika Patnaik [4], Susri Nayak [4], Kishore Dhara [5], Nimai Chandra Saha [6] and Caterina Faggio [7,*]

1 Department of Zoology, Sundarban Hazi Desarat College, Pathankhali 743611, India; s.saha.bgc.wbsu@gmail.com
2 National Environmental Standards and Regulations Enforcement Agency (NESREA), Osogbo 230, Nigeria; zubbydoo@gmail.com
3 Department of Zoology, S.B.S. Government College, Balurghat 733126, India; dip.m@rediffmail.com
4 Environmental Science Research Laboratory, Department of Zoology, Ravenshaw University, Cuttack 753003, India; lipika_pat@yahoo.co.uk (L.P.); sushree.nayak90@gmail.com (S.N.)
5 Freshwater Fisheries Research & Training Centre, Directorate of Fisheries, Kalyani 741235, India; kishordhara@gmail.com
6 Fishery and Ecotoxicology Research Laboratory, Department of Zoology, University of Burdwan, Burdwan 743611, India; prof.ncsahavcbu@rediffmail.com
7 Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98166 Messina, Italy
* Correspondence: cfaggio@unime.it

Abstract: Diazinon exposures have been linked to the onset of toxic pathways and adverse outcomes in aquatic species, but the ecological implications on model species are not widely emphasized. The objective of this study was to determine how the organophosphate pesticide diazinon affected hematological (hemoglobin, total red blood count, total white blood count, and mean corpuscular hemoglobin), growth (condition factor, hepatosomatic index, specific growth rate), biochemical (total serum glucose, total serum protein), and endocrine (growth hormone, tri-iodothyronine, and thyroxine) parameters in *Clarias batrachus* after chronic exposure. Diazinon was administered at predefined exposure doses (0.64 and 1.28 mg/L) and monitored at 15, 30, and 45 days into the investigation. Observation for most biomarkers revealed patterns of decreasing values with increasing toxicant concentration and exposure duration. Correlation analysis highlighted a significant inverse relationship between variables (mean corpuscular hemoglobin, condition factor, specific growth rate, tri-iodothyronine, thyroxine, and total serum protein) and elevated chronic diazinon exposure concentrations. The integrated indices (IBR and BRI) indexes were used to provide visual and understandable depictions of toxicity effects and emphasized the relativity of biomarkers in terms of sensitivity and magnitude or severity of responses under graded toxicant exposures. The significant damage reflected by evaluated parameters in diazinon exposure groups compared to control portends risks to the health of local fish populations, including *Clarias batrachus* in aquatic systems adjacent to agrarian landscapes.

Keywords: diazinon; *Clarias batrachus*; pesticide; chronic toxicity; hematological parameters; growth parameters; biochemical parameters; endocrine parameters

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1. Introduction

Pesticides, otherwise referred to as plant protection products (PPPs), by deliberate use and applications in pest control and crop protection have recorded elevated occurrences in the environment [1–3]. The rapid expansion of various types of integrated farming systems for better income and livelihood of farmers is also gaining importance across several developing climes [4]. Though integrated systems require fewer pesticides than the

conventional ones, they are used lavishly in co-culture farming techniques [5], the eventual contamination of aquatic ecosystems with PPPs either due to spray-drift, leaching, runoff, and/or accidental spills and from aquacultural applications [6–8] could culminate into risks of debilitating effects and mass mortalities of non-target species [9–15]. In addition, risks of sustained toxicity to local biota due to their persistence and high retention within environmental matrices is also a concern [3,16–19].

Diazinon (dimpylate) is an organo-thiophosphate derivative used widely as an agricultural and household insecticide. Although it is inactivated by photochemical oxidation and expected not to be persistent, it has an estimated moderate to high toxicity to freshwater fish, estuarine, and marine fish and potential high toxicity to birds [20]. Its documented high toxicity to bees and other beneficial insects [20] portends risks of ecological effects, including invertebrate taxa loss and trophic cascades [21], which could also impact the survival of local fish populations. Significant risks of cancer, lung lesions, and cytogenetic effects in humans under chronic or prolonged exposures to diazinon have also been demonstrated [22,23]. Model refinement and validation for diazinon PBPK/PD (physiologically based pharmacokinetic/pharmacodynamic) models using a series of in vivo pharmacokinetic and pharmacodynamic studies in the rat showed fairly rapid oral absorption followed by metabolism and distribution of the active oxon metabolites [24]. The toxic potential of this pesticide has been demonstrated using a number of biomarkers, including hematological changes (blood glucose, lipid, and serum enzyme profiles) and cross taxa histopathology [17,25–30]. In vitro studies on cell lines suggest that it causes oxidative stress through free-radical generation and promotes DNA fragmentation [31,32]. Thyroid disruption has been implicated in organophosphate exposures [33], while growth inhibition due to diazinon exposures has been reported [34].

Following reports of pesticides impacting and shaping the occurrence of global freshwater biodiversity, various criteria based on ecologically relevant risk estimates have been explored to protect aquatic life from pesticide stress [9,14,29,35–40]. Furthermore, the use of integrated biomarker endpoints in evaluating and visualizing chemical exposure effects on aquatic organisms is still not widely applied [41]. The present study sought to assess the chronic toxic impact of diazinon on hematological indices (hemoglobin, whole erythrocyte count, whole leucocyte count, mean corpuscular hemoglobin), morphometric indices (condition index, liver-body index, specific growth rate), endocrine indices (somatotrophic hormone, liothyronine, thyroxine) and biochemical indices (whole serum protein, whole serum glucose) of air-breathing catfish, *Clarias batrachus*. In addition, the gross effect of diazinon on fish using IBR (integrated biomarker response) as a summarizing index was also evaluated.

2. Materials and Methods

2.1. Experimental Organism

Fingerlings of the freshwater, air-breathing catfish *Clarias batrachus* were collected from a local fish farm in Basirhat, District North 24 Parganas, West Bengal, and transported to the Aquatic Toxicology laboratory, Barasat Government College, West Bengal (weight 7.9 ± 0.9 g (mean ± SD); length 7.5 ± 1.2 cm (mean ± SD)). Fish were conditioned to laboratory settings for three weeks in flow-through outdoor tanks (4000 L capacity) with dechlorinated water (pH 7.6–7.9) and ambient temperature under a natural photoperiod (12:12 h light-dark). Air pumps ensured that the tanks had a steady supply of oxygen (Despacito CT-202 Air Pump, Kolkata, India). The fish was served dry organic micro sinking pellets ad libitum (Growfin fish feed, manufactured by Growel feeds Pvt. Ltd., Andhra Pradesh, India). The fingerlings (n = 60) were kept for acclimation in an aquarium of 4000 L. Every two days, the water quality was maintained by a partial replenishment (30–35%) of the water [42].

2.2. Test Chemical

Technical grade (95% TC) diazinon (CAS no. 333-41-5; O, O-diethyl-O-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate; manufactured by King Quenson Group, Shenzhen, China) belonging to the organophosphate class of insecticide (molecular weight 304.34 g mol^{-1}) was used for the preparation of test solution.

2.3. Experimental Water

The bioassays and chemical analysis of the water were carried out following the American Public Health Association's guidelines [42]. Deep tube well water stored in an overhead tank was used as a diluent medium. Table 1 summarizes the physicochemical properties of water used for the control and test mediums throughout the experiment.

Table 1. Physico-chemical parameters of the control and test medium (0.64, 1.28 mg diazinon/L) used during the chronic toxicity study.

Parameters	15 Days			30 Days			45 Days		
	Dose (mg/L)								
	0.00	0.64	1.28	0.00	0.64	1.28	0.00	0.64	1.28
Temperature (°C)	28.2 ± 0.12	28.2 ± 0.15	28.1 ± 0.19	28.1 ± 0.21	28.3 ± 0.14	28.3 ± 0.21	28.3 ± 0.22	28.2 ± 0.28	28.7 ± 0.27
pH	7.5 ± 0.16	8.1 ± 0.14	8.3 ± 0.21	7.5 ± 0.19	8.4 ± 0.18	8.4 ± 0.18	7.7 ± 0.21	8.4 ± 0.16	8.4 ± 0.12
Dissolved Oxygen (mg/L)	5.5 ± 0.18	5.9 ± 0.22	6.1 ± 0.41	5.9 ± 0.36	5.9 ± 0.24	5.8 ± 0.22	5.9 ± 0.23	5.9 ± 0.43	5.9 ± 0.52
Nitrate (mg/L)	2.4 ± 0.12	2.5 ± 0.15	2.4 ± 0.10	2.4 ± 0.10	2.3 ± 0.06	2.5 ± 0.09	2.6 ± 0.10	2.6 ± 0.12	2.7 ± 0.13
Alkalinity (mg/L)	146 ± 2.20	208 ± 3.71	206 ± 0.43	145 ± 2.76	207 ± 3.43	207 ± 4.21	148 ± 2.89	207 ± 3.54	206 ± 3.24
Free CO_2 (mg/L)	13.2 ± 0.21	0.4 ± 0.12	0.9 ± 0.19	13.5 ± 0.32	0.4 ± 0.12	0.8 ± 0.19	13.9 ± 0.21	0.4 ± 0.23	0.7 ± 0.19
Hardness (mg/L)	114 ± 1.23	134 ± 1.76	140 ± 0.32	115 ± 2.37	135 ± 2.23	140 ± 1.75	115 ± 1.24	135 ± 1.65	138 ± 2.43
Ammonia (mg/L)	0.25 ± 0.02	0.29 ± 0.03	0.24 ± 0.04	0.29 ± 0.02	0.31 ± 0.04	0.33 ± 0.03	0.31 ± 0.02	0.32 ± 0.03	0.33 ± 0.01

2.4. QA/QC (Quality Assurance/Quality Control) Procedure

Diazinon was extracted using 1 mL C18 solid-phase extraction (SPE) columns. The conditioning of columns was performed in the sequence of 5 mL each of acetonitrile, methanol, and reagent water, maintaining a flow rate of 3 mL/min. A total of 100 mL of water sample was used for the extraction process. Air suction was applied to the column for 20 min, and diazinon was eluted with 20 mL methanol [43]. Diazinon was detected using gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies, Santa Clara, CA, USA; model 19091S-433) with an HP-5MS capillary column (25 m × 0.25 mm diameter × 0.25 mm thickness). The operating parameters used during the analysis were: MS detector interface (290 °C), injector temperature (260 °C), oven program, 80 °C (2.0 min), 1.0 µL automatic splitless injection, and ultrahigh-purity helium (grade 5) carrier gas previously programmed at a flow rate of 1 mL/min. The recovery percentage of diazinon was then estimated [43]. The nominal concentrations were used throughout the experiment as the difference between the nominal and measured concentrations was lower than 5%.

2.5. Experimental Design of Chronic Toxicity Study

The chronic toxicity experiments were conducted for 45 days with control and two treatments (0.64 mg/L and 1.28 mg/L), which corresponded to 1/20th (T_1) and 1/10th (T_2) of the 96h LC_{50} obtained for *C. batrachus* exposed to diazinon in our earlier work [44]. In a randomized design, acclimatized fingerling fish (n = 10) in experimental tanks (350 L capacity) were set up in four replicates [45]. To ensure the water quality and concentration of the test media, the medium in all the groups was completely replaced every five days with freshly made diazinon solution. For 45 days, the fish were fed three times a day until they were visually satisfied. Diazinon was detected using gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies; model 19091S-433) with an HP-5MS capillary column of 25 m × 0.25 mm diameter × 0.25 mm thickness following standard procedures [43]. The recovery percentage of diazinon was then estimated. The nominal concentrations were used throughout the experiment as the difference between the nominal and measured concentrations was lower than 15% for the different exposure periods. To

assay for hematological, biochemical, growth, and endocrine parameters, 3 individuals were sampled from each exposure concentration at days 15, 30, and 45.

2.6. Hematological Profiles and Indexes

To prevent stress, the fish were anesthetized with clove oil (60 µL/L water). For a 45 days exposure period, blood and serum were taken from both the control and treated fishes at 15 days intervals. Blood was obtained by puncturing the fish's caudal vessels with a 5 mL dispovan syringe that had been previously cleaned with a 4% EDTA solution. To avoid clotting, the blood was immediately transferred to vacutainer EDTA-coated tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and mixed by vertexing. In the absence of EDTA, a portion of the blood was taken and centrifuged for 20 min at 3000 rpm in a cooling centrifuge. The serum was taken with a micropipette, transferred to microtubes, and stored at −20 °C for further assessment of total serum protein (TSP), total serum glucose (TSG), growth hormone (GH), tri-iodothyronine (T_3), and thyroxine (T_4).

The hemoglobin level in blood was measured by Sahli's method using 0.1 N HCl. The total red blood count (TRBC, $10^6/mm^3$) and total white blood count (TWBC, $10^3/mm^3$) were measured following standard protocols [46,47]. The TRBC ($10^6/mm^3$), TWBC ($10^3/mm^3$), and mean corpuscular hemoglobin (MCH, pg) were estimated using the formula:

$$\text{TRBC } (10^6/mm^3) = [\text{Total number of cells counted in Neubauer Haemocytometer} \times \text{dilution factor (200)}]/[1/5 \times \text{volume factor (0.1)}]$$

$$\text{TWBC } (10^3/mm^3) = [\text{Total number of cells counted in Neubauer Haemocytometer} \times \text{dilution factor (50)}]/[4 \times \text{volume factor (0.1)}]$$

$$\text{MCH (pg)} = [\text{Hemoglobin (g/dL)} \times 10]/\text{TEC } (10^6/mm^3)$$

2.7. Growth Endpoints

This test was performed on a different group of fish that included both male and female fish. For the whole 45-day exposure period, the condition factor (K), hepatosomatic index (HSI), and specific growth rate (SGR) were assessed at 15-day intervals. The length and weight of each fish from the tank were measured using a meter scale and portable weighing scale at the termination time of every 15 days for K, HSI, and SGR. Liver weights were recorded to the nearest tenth of a milligram. The following are the different equations used to calculate the aforementioned parameters [48,49]:

$$\text{Condition Factor (K, g/cm}^3) = (W/L^3) \times 100 \text{ [W = Body weight of fish (g), L = Body length of fish (cm)]}$$

$$\text{Hepatosomatic Index (HSI)} = [\text{Liver weight of fish (g)/Body weight of fish (g)}] \times 100$$

$$\text{Specific Growth Rate (SGR, \%/day)} = [(\text{Natural logarithm of initial body weight of fish—Natural logarithm of final body weight of fish})/\text{Time interval}] \times 100$$

2.8. Endocrine (GH, T_3, and T_4) Endpoints

Growth hormone (GH) test analysis was carried out using heterologous competitive ELISA, as described by Lal and Singh [50]. ELISA kits provided by Creative Diagnostics Co. (New York, NY, USA) were used to measure plasma T_3 and T_4 levels.

2.9. Biochemical Total Serum Protein (TSP), Total Serum Glucose (TSG)

The total serum protein (TSP) was calculated using the technique by Lowry et al. [51]. Total serum glucose (TSG) was tested using a one-touch Easy Glucometer (Johnson and Johnson®, New Brunswick, NJ, USA).

2.10. Integrated Biomarker Response (IBR)

Integrated biomarker response (IBR) was calculated as specified by Beliaeff and Burgeot [52] and a modified equation formulated by Samanta et al. [53]. Data were first standardized, and the score value (S) was calculated using the equation. The score (S) value was calculated as $S = Y + |\min|$, where $S \geq 0$ and $|\min|$ is the absolute minimum value of Y of each biomarker. This was followed by computation of standardized Z value either as ($Z = y$ or $Z = -y$) signifying response of the toxicant as activation or inhibition.

$$S = Z + |\mathrm{Min}|$$

where $S > 0$ and $|\mathrm{Min}|$ indicate the minimum absolute value.

Finally, the IBR value was computed using the following equation:

$$\mathrm{IBR} = \sum_{i}^{n} \left(\frac{S_i \times S_{i+1}}{2} \right)$$

where S_i and S_{i+1} indicate the score values of two consecutive star plot points and 'n' indicates the corresponding radii number.

2.11. Biomarker Response Index (BRI)

The biomarker response index (BRI) for determining and considering the health status of the test model was performed, taking into consideration of a standard protocol with slight moderations [54]. Documented interpretations for BRI indicate that a score of 4 = healthy organism, 3–4 = minor detrimental effect, 2.75–3 = moderate unfavorable impact, 2.5–2.75 = sizable adverse effect, while <2.5 = seriously negative impact [55].

3. Results and Discussion

3.1. Hematological Profiles

Compared to the control fish, the exposed fish showed a significant reduction in Hb, TRBC, TWBC, and MCH values in groups with higher exposure concentrations (Figure 1). The decrease in Hb and TRBC levels observed in diazinon exposure groups could be related to the disrupted function of the hemopoietic system, erythrocyte destruction, or reduced synthesis in bone marrow [56,57]. The decrease in total leucocyte count observed in diazinon-exposed fish could also be attributed to hematopoietic system dysfunction [58]. Long-term exposure to organophosphate pesticides has been implicated in the hemato-toxicity effects and increased incidence of aplastic anemia in exposed animals through the defective maturational and functional status of different marrow cell lineages [59]. Depression of growth factor production by cells of the hematopoietic microenvironment and various cellular disturbances throughout the hematopoietic system could be attributed to pesticide-induced toxicity [59]. Haemato-toxic effects similar to this study have also been documented for other benthopelagic fish species [26,60,61].

3.2. Growth Endpoints

The fish subjected to sub-lethal doses of diazinon showed a concentration-dependent and time-dependent reduction in physiological state and retarded growth rate as reflected by the K, HSI, and SGR, respectively (Figure 2a–c). Due to varied underlying physiological processes and synchrony with environmental changes, growth provides a robust indicator of ecological risks, particularly for chronic scale exposures [62,63]. Condition factor is an individual-level biomarker of physiological condition and reflective of the health and fitness state of the fish as a product of interactions with environmental quality [64,65]. As such, a significantly lower K at elevated diazinon exposures (Figure 2a) is strongly depictive of physiological stress and loss of fitness for survival [66].

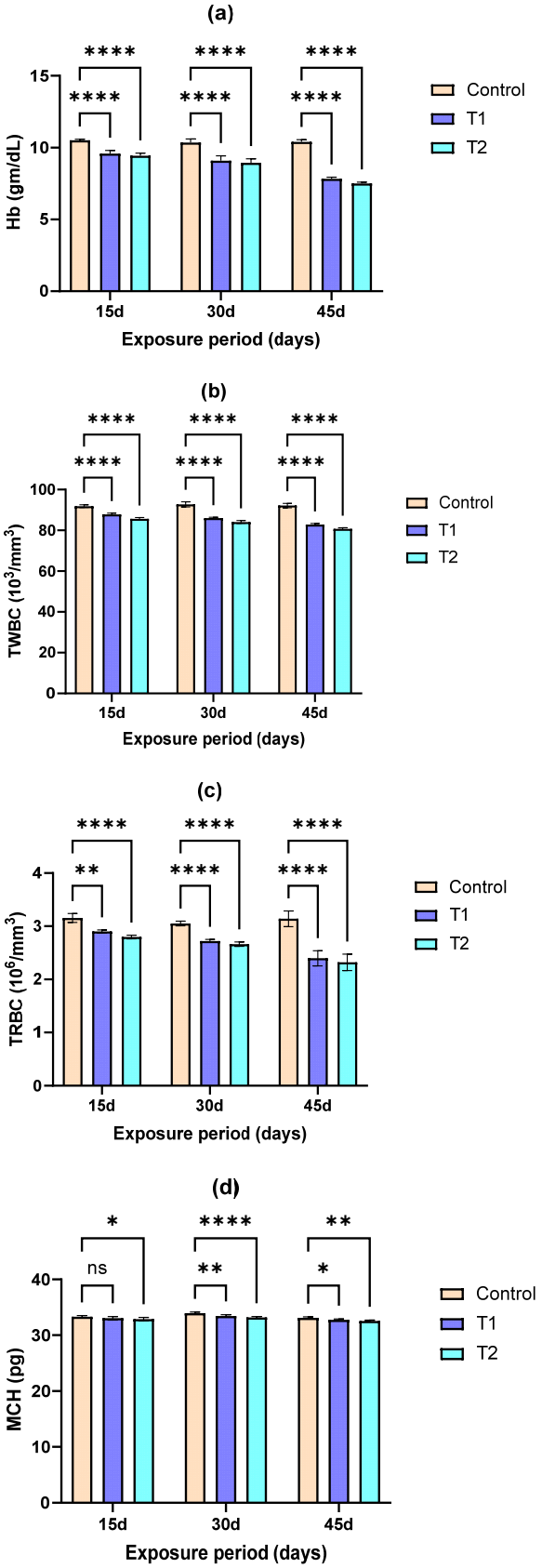


Figure 1. (**a**–**d**) Mean and SD values of hematological profiles and indices of *Clarias batrachus* exposed to different concentrations of diazinon (0.64 and 1.28 mg/L) for different exposure times (15, 30, and 45 days). *—denotes significant differences to control within the same exposure time (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$). (**a**) Hemoglobin; (**b**) total white blood cells; (**c**) total red blood cells; (**d**) mean corpuscular hemoglobin.

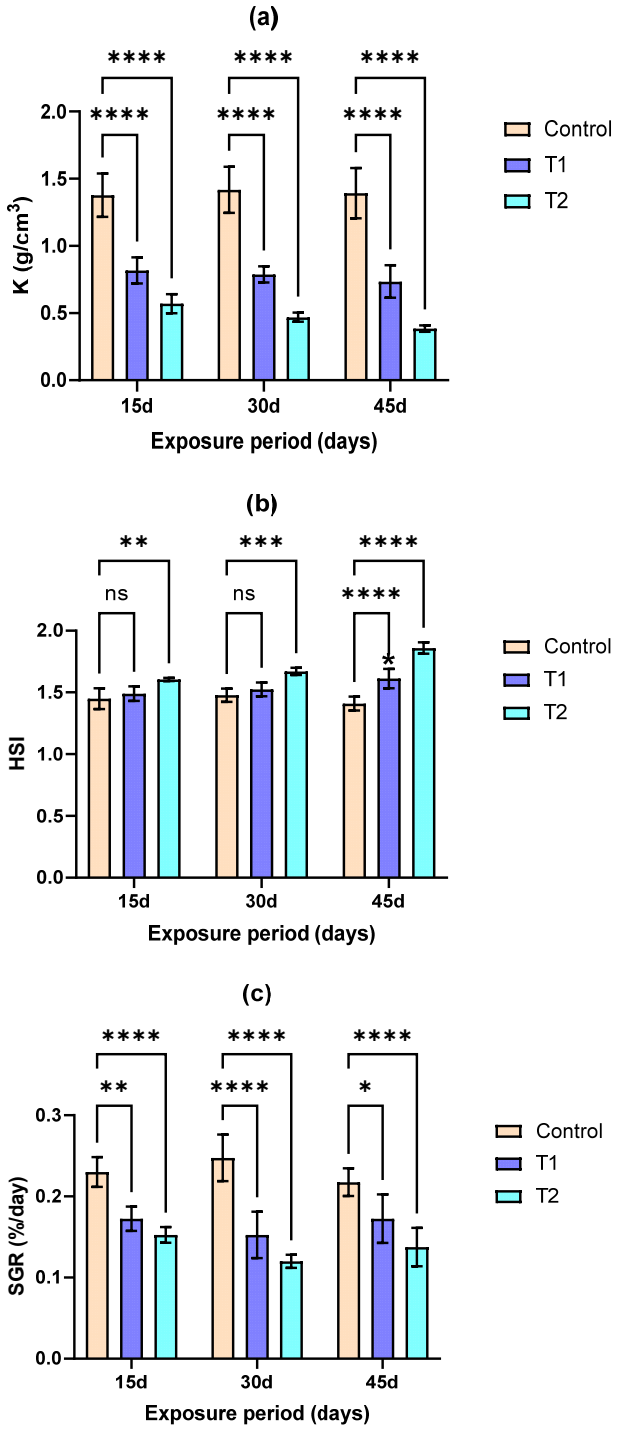


Figure 2. (**a**–**c**) Mean and SD values of growth endpoints and indices of *Clarias batrachus* exposed to different concentrations of diazinon (0.64 and 1.28 mg/L) for different exposure times (15, 30, and 45 days). *—denotes significant differences to control within the same exposure time (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$). (**a**) Condition factor (K); (**b**) hepatosomatic index (HSI); (**c**) specific growth rate (SGR).

Fish HSI values across diazinon exposure groups compared to control revealed significantly higher HSI values with increasing diazinon exposure concentrations ($p < 0.05$) (Figure 2b). Since the liver is the hub of metabolic activities, it is taken as a reliable predictor of toxic exposures and pathophysiological dysfunction [67,68]. Although several reports have attributed elevated HSI values to hepatomegaly, i.e., enhancement of the liver size

due to destructive changes [69,70], the enhancement of HSI may also reflect reduced body weight due to low food conversion [71].

Fish from other diazinon treatments (0.64 and 1.28 mg/L) displayed significantly lower SGR than in control groups ($p < 0.05$) (Figure 2c), indicating greater toxicity with increasing diazinon concentrations. This is consistent with previous research on various fish treated with multiple pesticides [72,73]. In this study, the specific growth rate of fish exposed to diazinon remained lower than control fish throughout the exposure intervals from 15 to 45 days. This unchanged significant difference between the SGR fish in the exposure groups and control highlights a possible inability to undertake compensatory growth response [74]. Although lowered SGR has been attributed to lower food intake and conversion [75], stress-induced chemical exposures have been implicated in the reduction in SGR [76], with endocrine disturbance as a possible mechanism [77].

3.3. Endocrine Endpoints (GH, T_3, and T_4)

The concentration of growth hormones (GH) and thyroid hormones (THs), T_3, and T_4 in *C. batrachus* from the control group were significantly higher compared to fish from the diazinon exposure group (Figure 3). Since several endocrinological factors regulate fish growth and development, the relative levels of this relative hormone detected across control and exposure groups will have implications for growth and development for the fishes [77–79]. A similar lowered growth hormone (GH) activity in pesticide exposed fish has been documented [80]. The reduced activity of T_3 and T_4 in *C. batrachus* from exposure groups highlights the disruptive endocrine properties of diazinon with implications for thyroid hormone signaling and metabolism in the hypothalamic-pituitary-thyroid (HPT) axis [81]. The HPT axis regulates the thyroid endocrine system by coordinating the synthesis, secretion, transport, and metabolism of thyroid hormones, and exposures to environmental chemicals such as pesticides can affect this axis, impairing the expression of several hormones along the HPT axis, including thyroid hormones [81,82]. Furthermore, since thyroid hormones (tri-iodothyronine, T_3, and thyroxine, T_4) are also crucial regulators in fish growth, metabolism, reproduction, and behavior [83], lowered expression will negatively impact normal ecological functions and survival of the affected fish population in the wild.

3.4. Total Serum Protein (TSP) and Total SERUM Glucose (TSG)

The changes associated with TSP and TSG levels are summarized in Figure 4. Concentration-dependent depletion of TSP and elevated levels of TSG were observed in diazinon-exposed *C. batrachus* compared to the control group (Figure 4). The significantly lower levels of complete serum protein in diazinon-exposed fish implicate increased energy demand typical of chemical-induced stress and toxicity [84,85]. Similar patterns of lowered serum protein levels have been documented in other fish species exposed to diazinon [86].

Also, in this study, the dramatically higher blood glucose levels in diazinon-exposed *C. batrachus* highlight toxicity-related energy demand necessitating increased mobilization of glycogen into glucose for biotransformation of xenobiotics and sustaining compensatory responses. Under stress, adrenal tissue secretes more glucocorticoids and catecholamines, resulting in hyperglycemia [26]. Elsewhere, hyperglycemia following diazinon exposure has been attributed to limiting glucose metabolism due to a reduction in AChE (acetylcholinesterase) activity [87]. In addition, diazinon-induced oxidative damage in the liver and pancreas of the treated organism may also disrupt insulin and glucose homeostasis [88].

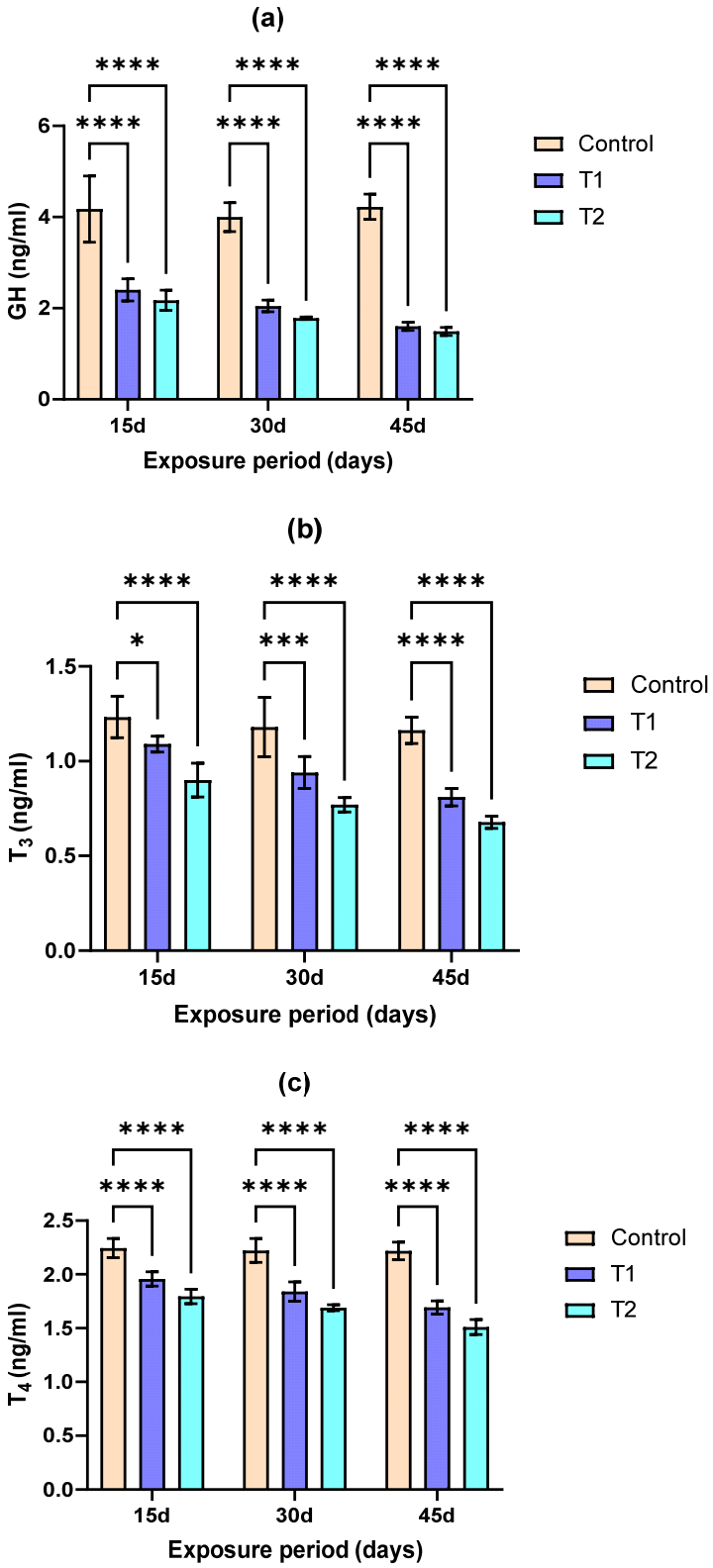


Figure 3. (**a**–**c**) Mean and SD values of different hormones of *Clarias batrachus* exposed to different concentrations of diazinon (0.64 and 1.28 mg/L) for different exposure times (15, 30, and 45 days). *—denotes significant differences to control within the same exposure time (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$); GH—G=growth hormone, T_3—tri-iodothyronine, T_4—tetra-iodothyronine (thyroxine).

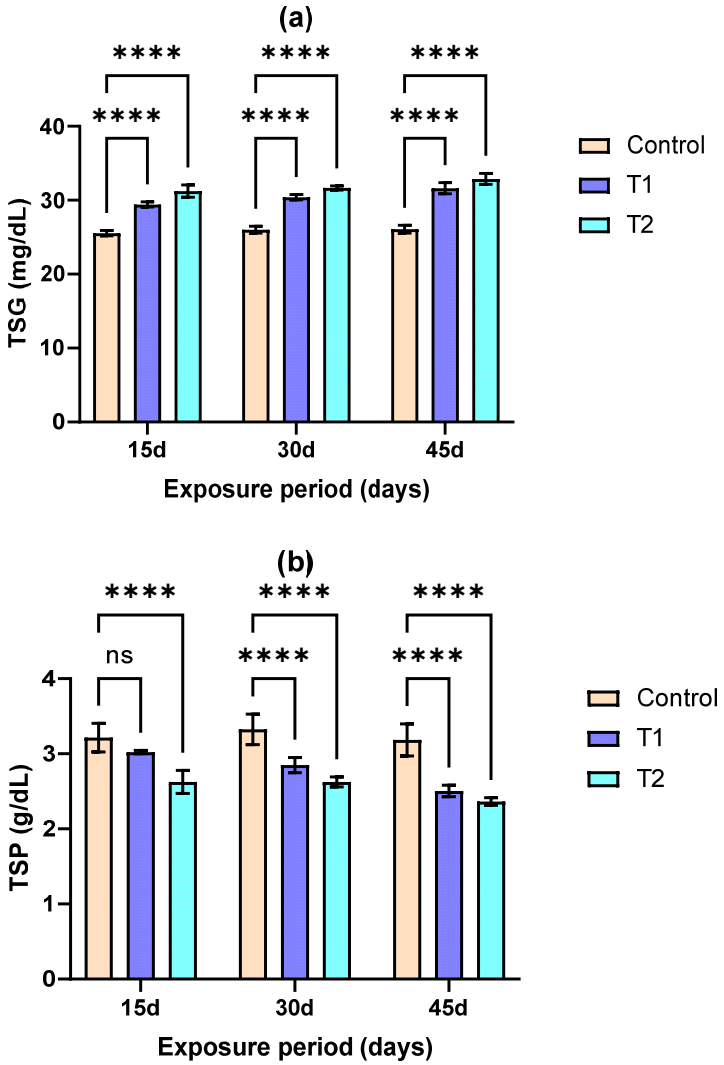


Figure 4. (**a**,**b**) Mean and SD values of different biochemical parameters of *Clarias batrachus* exposed to different concentrations of diazinon (0.64 and 1.28 mg/L) for different exposure times (15, 30, and 45 days). *—denotes significant differences to control within the same exposure time (**** $p < 0.0001$); TSG—total serum glucose, TSP—total serum protein.

3.5. Correlation Analysis

Correlation analysis between exposure concentrations and hematological, growth, endocrine, and biochemical indices is given in Figure 5. Exposure concentration showed the strongest negative correlation with MCH, K, SGR, T_3, T_4, and TSP, indicating that the values of these parameters reduced from the control group to the highest exposure concentration. In addition, exposure concentration showed a strong positive correlation with HSI and TSG, indicating that the values of these parameters increased from control to the highest exposure concentration. While toxicology studies have demonstrated the disruptive effects of pesticides on erythropoietic tissues [89–93], the relatively higher sensitivity of MCH compared to other hematological parameters in fish at elevated toxicant exposure concentrations have been reported [94]. The significant reduction in MCH across exposure groups ($p < 0.05$) when compared with the control could be attributed to lowered levels of cellular blood iron or heme-synthesis dysfunction, which stresses the fish by reducing the oxygen-carrying capacity of its blood [89,95]. The negative correlation between exposure concentration and the K condition factor also reflects a deteriorating physiological condition of exposed fishes with chronic higher toxicant concentrations. In particular, lowered condition factor highlights disrupted food intake of lowered conversion

of food intake into tissue development [64]. A low body condition may also suggest muscle wasting (proteolysis), indicating a starvation response [96,97].

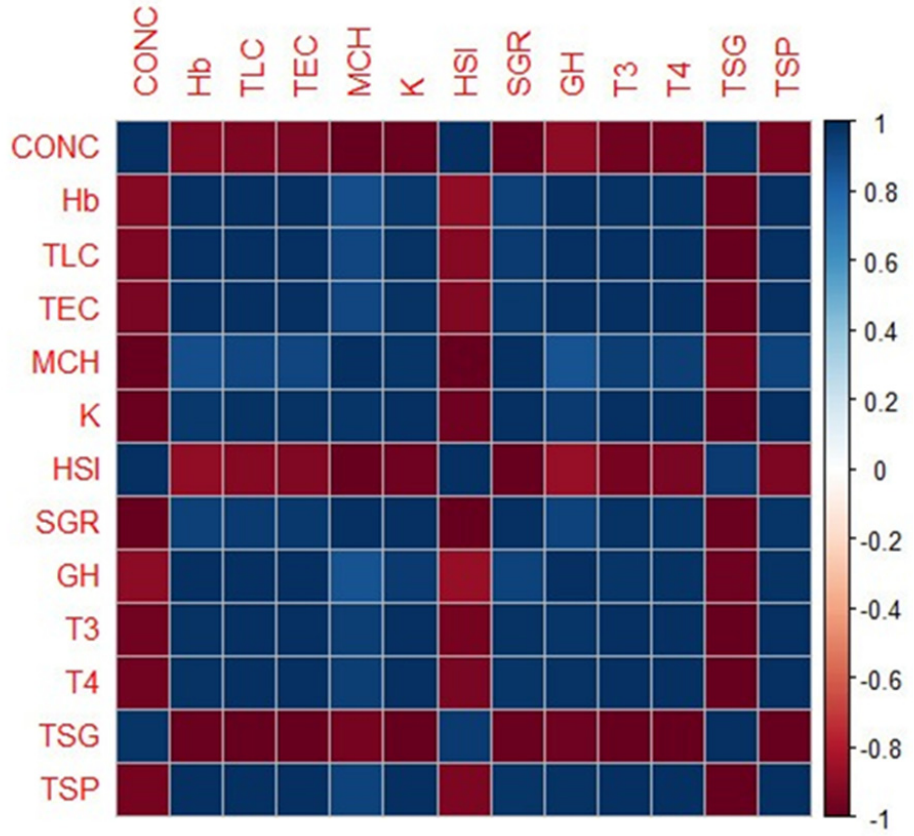


Figure 5. Correlogram of exposure concentration with hematological, biochemical, and growth parameters in *Clarias batrachus*.

The strong negative correlation between exposure concentration and specific growth rate (SGR) indicates decreased growth also attributable to impaired fish feeding activity [98]. The significant inverse correlations between diazinon exposure concentrations and T_3, T_4 levels in the fish highlight the endocrine disruptive potential of diazinon under chronic-exposure regimes. This endocrine disruption will, in turn, impact the growth and development of exposed fishes. Thyroid hormones are essential for somatic growth and for early development in fishes, including larval–juvenile transitions and induction of metamorphosis [99–101]. The significant inverse relationship between exposure concentration and total serum protein also reflects its sensitivity to toxicant-related stress after the onset of physiological effects. Changes in serum TP (total protein) may be due to liver damage, reduction absorption, and protein loss and thus may be a suitable indicator of the health status of fish [102]. Retarded growth responses alongside a reduction in serum protein with elevated diazinon exposures in this study also give credence to the possibility that the lowered condition factor and specific growth rate is caused by an underlying starvation response and muscle wasting (proteolysis) [96,97].

The strong positive relationship between diazinon exposure concentrations and TSG indicates that glucose content was lowest in control and increased in diazinon chronic-exposure groups. This strongly highlights remnants of ongoing physiological-level compensatory reactions to out-with the effects of the toxicant. Prior elevation of serum glucose level and eventual decline until the depleted level was attained has been reported for prolonged exposure to toxicants [103,104]. Thus, the higher levels in diazinon-exposed groups reflect not-yet depleted energy reserves to cope with diazinon-related exposure and uptake. The strong positive correlation between exposure concentration and HSI

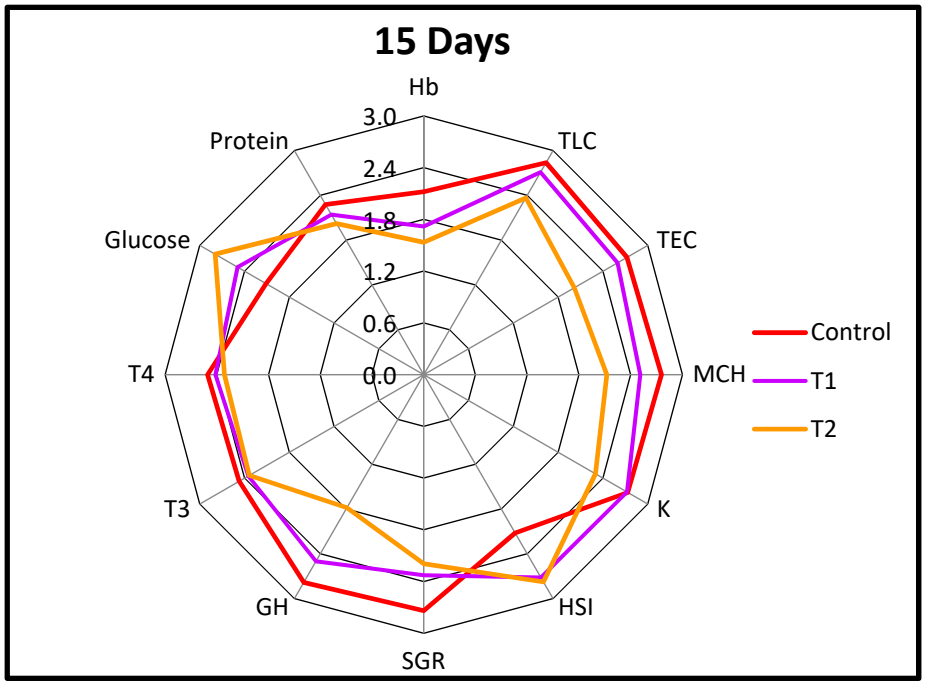
also indicates increased values of this parameter from the control group to the diazinon chronic-exposure groups. The strategic use of HSI as an index of exposure to contaminants is largely attributed to the underlying role of the liver in the metabolic detoxification and biotransformation of pollutants in fish [105]. A higher HSI in toxicant-exposed groups has been linked to histopathological changes in hepatocytes, i.e., cell enlargement (hypertrophy) and/or increasing the number of hepatocytes (hyperplasia) in exposed fish [106].

3.6. Integrated Biomarkers Response (IBR)

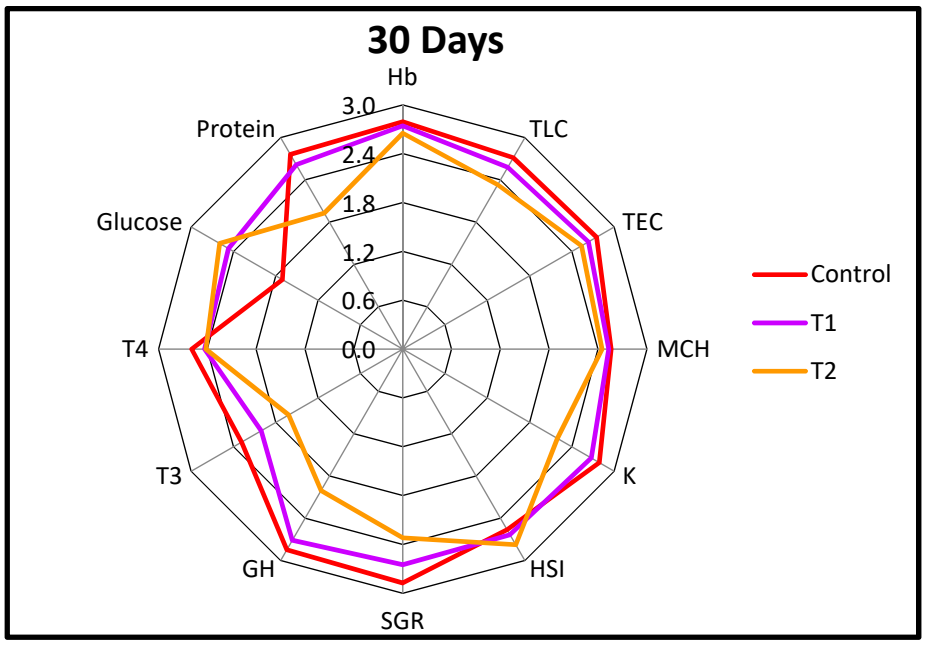
The score values of the various studied parameters in the blood of diazinon-exposed *Clarias batrachus* have been represented as star plots in Figure 6a–c, for a period of 15, 30, and 45 days, respectively. The score values for parameters such as Hb, TLC, TEC, MCH, K+, SGR, GH, T_3, T_4, and protein decreased with the increasing concentration of the toxicant. A decreasing trend was observed for all the exposure periods. The score values for HSI and glucose increased with an increase in dose concentrations. A similar increasing trend was observed with periods of toxicant exposure. The IBR values for different dose concentrations and periods were calculated using the score values and represented graphically in Figure 7. The IBR values for the control and experimental group for 15 days of exposure was estimated to be 36.93, 32.67 (T_1), and 33.26 (T_2). IBR values for 30 days of exposure to diazinon were calculated to be 40.12 (control), 35.75 (T_1), and 36.15 (T_2). Similarly, IBR values for 45 days were 38.79 (control), 36.19 (T_1), and 36.62 (T_2), respectively. These marked depictions of relative toxicity using changes of each endpoint on the star plot reflect a major advantage of IBR as a technique in toxicity assessments. The visual feature of IBR highlights its strength as a species-specific and toxicant-specific technique that affords a ready qualitative assessment across parameters and groups [107]. The overall effects of various xenobiotics in different groups of animals, including invertebrates, have been demonstrated using this technique [108,109]. Aside from the simplification of complex biological responses into a single index and predefined quality class, the IBR chart also simplifies relative toxicity under exposure gradients [54,110].

3.7. Biomarker Response Index (BRI)

Biomarker weights and scores were calculated for the studied parameters of the exposed fish and integrated to calculate and estimate the BRI. BRI is indicative of the general health status of the fish [54]. In the present study, the BRI value of diazinon-exposed fish for 15 days was 3.42 for T_1 and 3.25 for T_2, which indicates slight alterations in the treated fishes' health status compared to the control [54]. The BRI value for 45 days was three, which reflects moderate alteration compared to the control group. The BRI value of diazinon was 2.75, which specifies significant modifications of the health status compared to the control [54]. Overall, BRIs exhibited significantly concentration-effect responses, showing an apparent decrease with the increasing diazinon exposure levels. In addition, the BRI appeared to show marked differences compared to the IBR. The advantage of this integrated index obtaining an accurate toxicity scale has been reported [111].

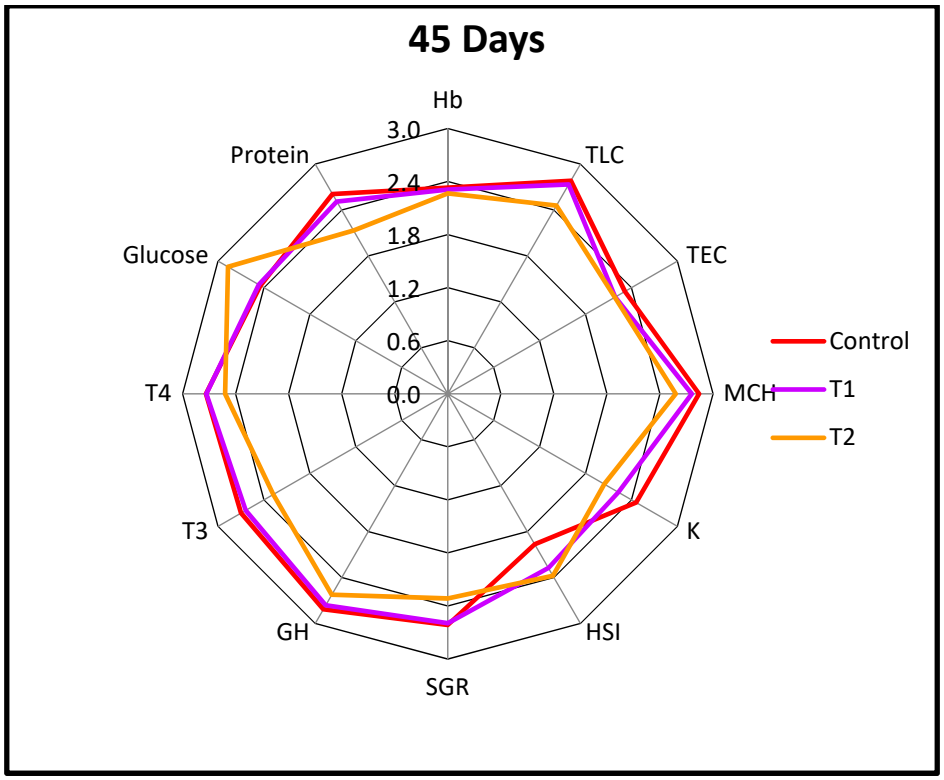


a



b

Figure 6. *Cont*.



c

Figure 6. Star plot of the studied biomarkers in *Clarias batrachus* after (**a**) 15 days, (**b**) 30 days, and (**c**) 45 days diazinon exposure.

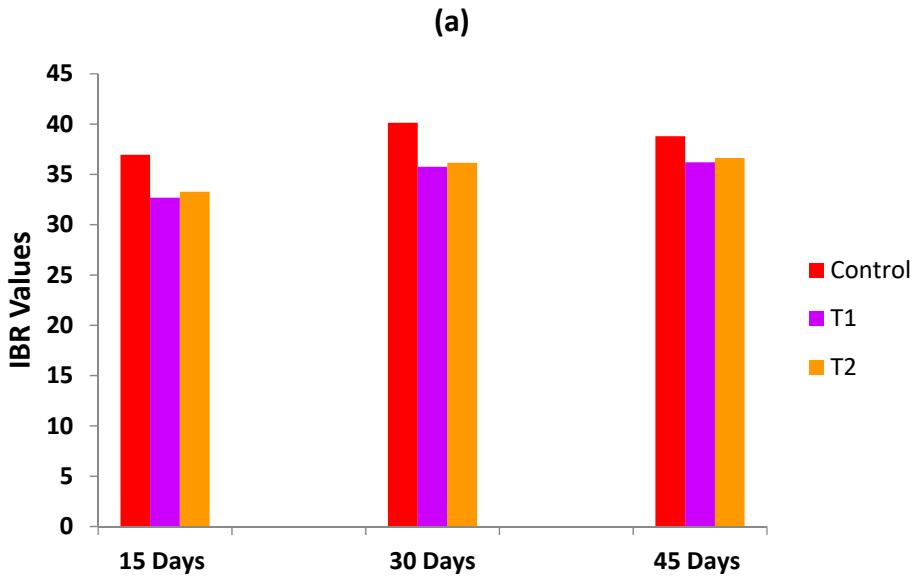


Figure 7. *Cont.*

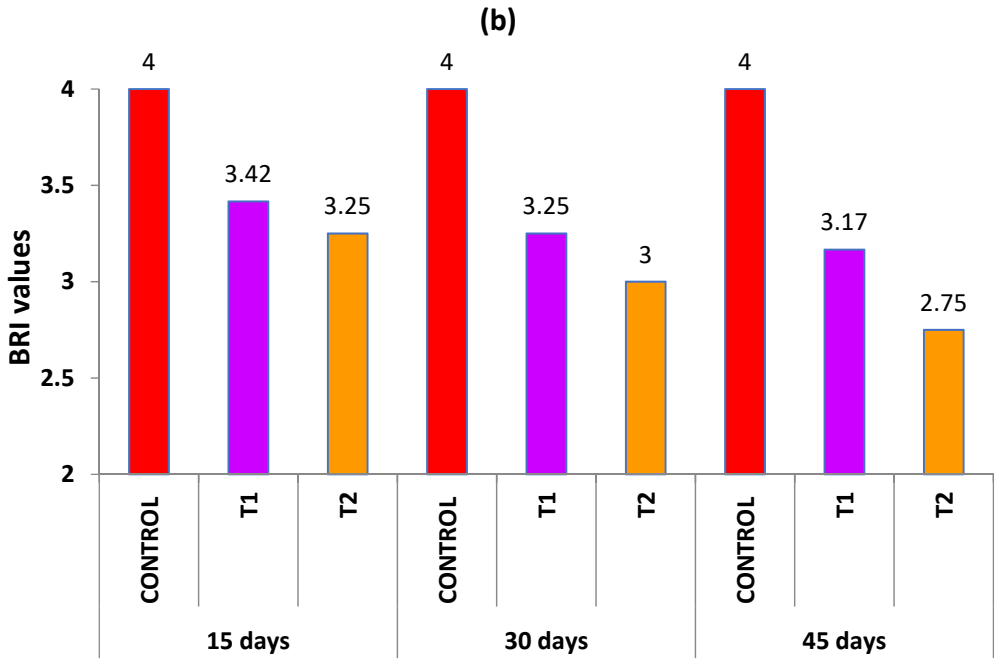


Figure 7. (**a**) IBR values of biomarkers in *Clarias batrachus* exposed to diazinon for different periods (**b**) BRI values of test fish upon exposure to diazinon for 15, 30, and 45 days.

4. Conclusions

The observation for most biomarkers revealed patterns of decreasing values with increasing toxicant concentration and exposure duration. The capacity for diazinon to cause haematoxicity, endocrine disruption, growth retardation, and biochemical modulations under chronic exposures has been demonstrated in this study. The integrated indices (IBR and BRI) indexes were used to provide visual and understandable depictions of toxicity effects and emphasized the relativity of biomarkers in terms of sensitivity and magnitude or severity of responses under graded toxicant exposures. The significant damage reflected by evaluated parameters in diazinon exposure groups compared to control portends risks to the health of local fish populations, including *Clarias batrachus* in aquatic systems adjacent to agrarian landscapes.

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Data Availability Statement: Data are contained within the article.

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