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Toxic Effects of Fungicide Sheathmar to Worm, *Tubifex Tubifex*

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Abstract: ***The current study sought to determine Sheathmar's acute toxicity as well as its sublethal effects on oxidative stress enzymes in Tubifex tubifex, a benthic oligochaete worm. The results showed that the 96-hour median lethal concentration (LC_{50}) of Sheathmar for Tubifex tubifex is 3186.3 µg/l. According to the model fit performance, the GUTS-SD model can better predict the survival rate of Tubifex tubifex. Sublethal Sheathmar concentrations (10% and 20% of the 96h LC_{50}) significantly changed the oxidative stress enzymes. Reduced glutathione (GSH), glutathione S-transferase (GST), and glutathione peroxidase (GPx) activity all showed a significant initial increase followed by a decline, whereas catalase (CAT) activity and malondialdehyde (MDA) levels increased significantly with increasing Sheathmar concentrations at all exposure times. Furthermore, the effects of Sheathmar on Tubifex tubifex were demonstrated by the development of a potency index and the assessment of integrated biomarker response (IBR). These findings suggest that exposure to Sheathmar influences Tubifex tubifex survival at the acute stage and modifies oxidative stress enzyme alterations at the sublethal level.***

Keywords: Sheathmar, Tubifex tubifex, acute toxicity, oxidative stress, Pearson correlation matrix, integrated biomarker response

I. INTRODUCTION

In recent years, the rapid increase in human population and the rapid pace of industrialization have created a food grain production problem [1]. Crop damage caused by various pathogens such as insects, weeds, fungus, bacteria, viruses, and so on is another major issue [2]. However, the continuous and extensive application of pesticides frequently results in environmental water contamination and has a negative impact on aquatic organisms [3], [4]. Pesticides are designed to kill, repel, and reduce pests, unwanted herbs, rodents, fungi, or other organisms that pose a threat to crop plants [5]. As a result, they are widely used by farmers in modern agriculture practises to increase crop production in order to sustain the human population. However, a lack of knowledge and injudicious pesticide use results in lethal effects on organisms [6]-[8]. These toxicants enter the aquatic system via surface runoff and endanger non-target organisms [9]. These toxic chemicals alter the quality of water, affecting the health of various aquatic organisms [10]-[12].

Tubifex tubifex is a benthic oligochaete worm that lives in freshwater sediments. It is a massive species with a global distribution that can withstand a variety of environmental conditions. It is easily grown in laboratories and is an important food source for fish [13]. While preliminary toxicity research uses a lethal endpoint such as the LC_{50}, sublethal toxicity studies are far more prudent because the species is exposed to much lower, biologically relevant hazardous quantities of toxic compounds [14]-[17]. Furthermore, the use of general unified survival models (GUTS) has been suggested as an appropriate strategy for assessing toxicant risk in the environment. Two survival strategies define the damage-related mortality process: stochastic death (SD) and individual tolerance (IT).

In the SD model, individuals are comparable, and the risk of death from chemical stress increases as damage increases when a certain level of impairment is reached. Individuals, on the other hand, vary in their susceptibility to chemical stress, and once the damage exceeds a certain threshold, the individual dies instantly [18], [19]. Xenobiotic metabolism in organisms contributes significantly to the formation of reactive oxygen species (ROS) [20]. ROS effectively initiate lipid peroxidation (LPO) and cause severe oxidative stress damage to biomolecules such as DNA, proteins, and membranes [21]. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and their neutralisation by antioxidant enzymes such as CAT, SOD, GPx, and GSH [22]. As a result, an efficient secondary technique for assessing antioxidant enzyme activity may be useful in aquatic toxicology research [23].

A few observations on oxidative stress changes in *Tubifex tubifex* after pesticide exposure have been presented [24]-[29]. However, there is little research on pesticides' negative effects on oxidative stress in these worms. Because single biomarkers cannot provide an accurate and practical assessment of a toxicant's toxicity on aquatic life forms, an amalgamated biomarker analysis is advised to better understand an organism's response to toxic substances [30]. As a result, IBR provides a comprehensive methodology that incorporates all biomarker reactions and is critical in determining contaminant toxicity [31], [32].

As a result, the goal of this study is to assess Sheathmar's acute toxicity to *Tubifex tubifex* in terms of LC_{50} values after acute exposure, as well as to investigate Sheathmar's possible toxicity at sublethal concentrations by monitoring changes in oxidative stress indicators. The toxicity of Sheathmar in *Tubifex tubifex* is then determined using IBR. The GUTS-SD and IT models were used to predict toxicity, assess aquatic species' acute responses to pesticides, and determine which model, SD or IT, best matched the toxicity data.

II. MATERIALS &METHODS

The US EPA-specified quality assurance procedures for sample processing, storage, and preservation were followed.

A. Test Organism and Maintenance Condition

Adult *Tubifex tubifex* (Phylum: Annelida, Class: Clitellata, Order: Oligochaeta, and Family: Naididae) were collected from a local aquarium shop in Burdwan, West Bengal, India and acclimatized in unchlorinated water for 24 h (temperature 25.9 ± 0.4 °C, pH 7.2± 0.6, free CO_2 16.9 ±0.7 mg/l, dissolved oxygen 7.1 ±0.5 mg/l). Then, organisms averaging 12.5 ± 0.2 mm in length were added to the experimental setup. The physiochemical characteristics of the test water were maintained during the exposure duration (temperature 28.2 ±0.2 °C, pH 7.8 ±0.3, free CO_2 19.5 ±0.3 mg/l, dissolved oxygen 5.8 ±0.5 mg/l, total alkalinity 187 ±5.2 mg/l as $CaCO_3$, hardness 110 ± 4.1 mg/l as $CaCO_3$).

B. Test Chemicals

Sheathmar technical grade was obtained from the market through Sisco Research Laboratories Pvt. Ltd. (SRL), India. The Sheathmar stock solution and subsequent dilutions were prepared according to a standard protocol [33].

C. Bioassay for Acute Toxicity and Survival rate Projection

In 250 mL glass beakers containing 200 mL water and ten *Tubifex tubifex*, a static renewal acute toxicity bioassay was performed. Each experiment was carried out three times. To determine the range of mortality levels, a range detection test was first performed. Following that, the worms were exposed for 96 hours to various nominal concentrations of Sheathmar (00, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500, 4750, 5000 µg/l) with a control containing water free of the toxicant. At 24, 48, 72, and 96 hours, the worms were counted for mortality. Finney's probit analysis was used to calculate the LC_{50} values at 24, 48, 72, and 96 hours, with log concentration as the dependent variable and probit as the independent variable [34]. *Tubifex tubifex* survival rate pattern in response to Sheathmar was evaluated using GUTS modelling, which was accomplished using the standalone software OpenGUTS. k_d (the dominant rate constant), m_w (the median of the threshold distribution), h_b (the background hazard rate), and b_w (the killing rate that is exclusively used for SD) are the model parameters employed [18], [19].

D. Determination of Oxidative Stress Parameters at Sublethal Levels

2g of *Tubifex tubifex* is transferred from the stock tank to glass beakers, each holding 1 litre of unchlorinated tap water, to analyse oxidative stress enzyme parameters at a sublethal level. Two sublethal concentrations of Sheathmar were administered over 1d, 7d, and 14d (10% of 96h LC50 values, i.e., 318.6 µg/l and 20% of 96h LC_{50} values, i.e., 637.26 µg/l). The control worms were placed in another glass beaker with 1l of sterile, toxicant-free water. Sheathmar was introduced into the experiment on day one (initial treatment). The test medium was then replaced with Sheathmar at 10% of the initial nominal concentration every two days. During the exposure times, continuous aeration was provided. The procedure was repeated three times. At each exposure period, 1 g of worms were collected and homogenised in a 0.1 M phosphate buffer (pH 7.6). A cold centrifuge (Hermle Labortechnik) was used to perform a 10-minute centrifugation at 10000 g, and the supernatant was stored at -200 C until further analysis. The protein content was determined using the Bradford method [35]. Standard techniques have been utilized to quantify the activities of CAT (Beers and Sizer, 1952), SOD [37], GST [38], GPx [39], MDA [40], and GSH [41]. The effects of CAT, SOD, GSH, GST, and GPx were quantified in units per milligram of protein (U/mg protein). MDA levels, on the other hand, were measured in terms of nanomoles of thiobarbituric acid reactive substance (TBARS) per minute per milligramme of protein (nmol TBARS/min/mg protein).

E. Determination of IBR

The data on oxidative stress biomarkers were articulated and expressed in radar plots using an IBR system based on the standard protocol of [42].

F. Statistical Analysis

Finney's probit analysis in Microsoft Excel 2013 was used to calculate the LC50 values. Kaplan-Meier analysis was used to create survival curves. The comparisons between controls and exposed worms were identified using a two-way ANOVA followed by the Tukey post hoc test. The results of the analyses are summarised as mean standard deviation. Mean values with a significance level of $p<0.05$ are considered statistically significant. The associations between oxidative stress indicators were determined using a correlation matrix plot.

III. RESULT AND DISCUSSION

Table 1 shows the LC_{50} values for Sheathmar to *Tubifex tubifex* with 95% confidence intervals, which are 3921.75, 3695.19, 3441.09, and 3186.3l µg/l, respectively.

Table 1: Lethal concentration values and 95% confidence limits of Sheathmar to *Tubifex tubifex*

Point	Exposure concentration (µg/l)			
	24h	48h	72h	96h
LC_{50}	3921.75	3695.19	3441.09	3186.3

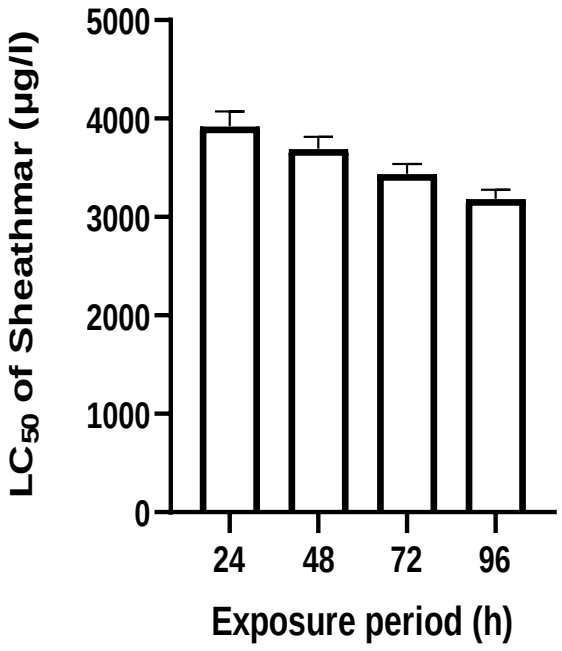
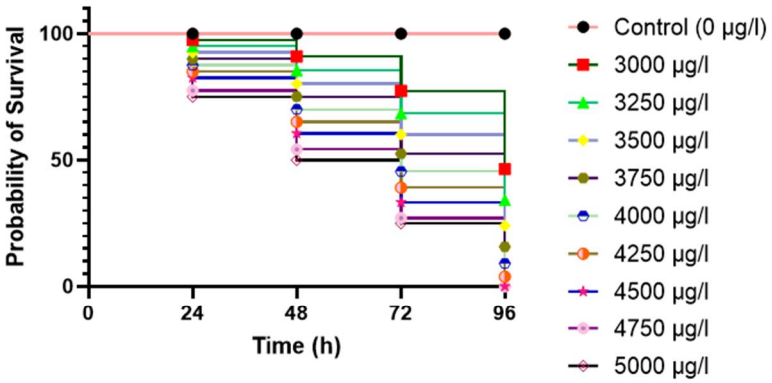


Fig 1: LC_{50} graph of *Tubifex tubifex* exposed to different concentrations of Sheathmar (00, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500, 4750, 5000 µg/l) at different exposure periods (24, 48, 72 and 96 h).

Sheathmar is thus considered moderately toxic to *Tubifex tubifex* based on the LC_{50} values. Furthermore, the survivability curve shows that Sheathmar had a significant effect on the overall survival rates of *Tubifex tubifex* in a dose and duration-dependent manner in comparison to the control (Mantel log-rank test; $p < 0.05$). (Fig 1). *Tubifex tubifex* is found to be 100% survivable in all exposure periods (24, 48, 72 and 96 h).



Log-rank (Mantel-Cox) test (recommended)	
Chi square	99.44
df	9
P value	<0.0001
P value summary	****
Are the survival curves sig different?	Yes

Fig 2: Kaplan-Meier survival curves of *Tubifex tubifex* exposed to different concentrations of Sheathmar (00, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500, 4750, 5000 µg/l) at different exposure periods (24, 48, 72 and 96 h).

However, as Sheathmar concentrations and exposure times (24, 48, 72, and 96 h) increased, the survivability rate of *Tubifex tubifex* decreased significantly (Mantel log-rank test; p 0.05). Table 2 shows the model parameters as well as the fitted performance of GUTS (SD or IT). Based on AIC values, the fitted performance of GUTS-IT was better than that of GUTS-SD in the case of Sheathmar (a smaller AIC value indicates the best fit). At an acute level, the model simulation demonstrated that the GUTS-IT model could better predict the survival rate observed in *Tubifex tubifex* for pesticide exposure than the GUTS-SD model. The survival model output shows that when determining the toxic effects of various toxicant exposure patterns, the model deducing SD or IT should be chosen wisely. It is obvious that such mechanistic modelling has a significant future potential for improving the accuracy of environmental risk management and can significantly aid in effective decision-making. The 100d LC_{50} value was calculated using the GUTS-IT model and is shown in Tables 2 and 3.

Table 2. Model parameters in case of Sheathmar [Kd indicates Dominant rate constant; m_w indicates Threshold for mortality; b_w indicates Killing rate; h_b indicates Background hazard rate & F_s indicates Spread factor of the threshold distribution]

Symbol	GUTS-RED		unit	AIC Value	
	SD	IT		SD	IT
k_d	143.8 (11.81 - 143.8)	1.276 (1.022 - 1.526)	d^{-1}		
m_w	2210 (2063 - 2246)	3058 (2811 - 3276)	µg/l	276.53	291.98
b_w	0.0002859 (0.0002203 - 0.0003606)	-	L/µg/d		
h_b	$1E^{-6}$	$1E^{-6}$	d^{-1}		
F_s	-	1.775 (1.537 - 2.276)			

Moreover, the forecasted LC_{50} values from GUTS-IT models are given in **Table 3**.

Table 3. The forecasted LC_{50} values of Sheathmar to *Tubifex tubifex*

Time [d]	LC_{50} GUTS-SD (µg/L)
1	4242 (3948 - 4643)
2	3316 (3121 - 3529)
3	3126 (2905 - 3335)
4	3077 (2835 - 3296)
7	3058 (2801 - 3285)
14	3058 (2800 - 3285)
100	3058 (2800 - 3285)

Antioxidant enzymes are direct oxidative stress biomarkers, capable of neutralising reactive oxygen species (ROS) and other prooxidative enzymes in cells under normal conditions [43], [44]. Figure 2 depicts the effect of Sheathmar on various antioxidant enzymes. CAT is a critical enzyme in the antioxidant system that effectively neutralises reactive oxygen species (ROS) and degrades H_2O_2 to molecular oxygen and water [45], [46]. Catalase activity increased significantly at 318.6 µg/l and 637.26 µg/l of Sheathmar during the 1, 7, and 14 d exposure periods (p 0.05). Increased CAT expression leads to increased nuclear Nrf2 expression, which protects cells from H_2O_2-induced stress [47]. Yile Yu et al. (2021) discovered a consistent increase in CAT activity in *Tubifex* following Dufulin exposure [30]. SOD is the most important oxidative stress enzyme because it converts reactive oxygen radicals to hydrogen peroxide, which provides significant resistance to oxidative stress [48]-[51]. SOD activity increased significantly at 318.6 µg/l and 637.26 µg/l of Sheathmar during the 1 and 7 d exposure periods, but decreased significantly at 318.6 µg/l and 637.26 µg/l of Sheathmar during the 14d exposure period ($p<0.05$). This increase in SOD activity could be attributed to superoxide ion stimulation, which activates the formation of SOD, which protects cells from oxidative damage [52]. The decrease in SOD activity on day 14 is most likely due to the excessive formation of ROS as a result of toxic pollution, which harmed or inactivated SOD's action by oxidising the cysteine in SOD or by reducing the expression of SOD-related genes [53]. GPx reduces oxidative stress by hastening the conversion of hydrogen peroxide to water and oxygen. When GPx is blocked, more hydrogen peroxide is available, which causes tissue degradation and oxidative stress. GSH concentration is always specifically linked to GPx activity. This is because it promotes the synthesis of oxidised glutathione by removing hydrogen peroxide with reduced glutathione [54]. GPx activity increased significantly on days 1 and 7 at all Sheathmar concentrations (318.6 µg/l and 637.26 µg/l, respectively) and appeared to play a significant role in antioxidant protection [55]. GPx activity decreased significantly compared to the control level at all Sheathmar concentrations (318.6 µg/l and 637.26 µg/l, respectively) at the end of the 14-day exposure period ($p<0.05$). This decrease in GPx could be due to the antioxidant defence system's inability to prevent toxicant-induced ROS generation [56]. This decrease could mean that the amount of hydroperoxide produced during lipid peroxidation exceeds the antioxidant capacity [57]. Furthermore, changes in GPx activity are most likely caused by changes in GPx mRNA expression [58]. *Tubifex tubifex* showed similar results after organophosphate pesticide profenofos, pyrethroid pesticide λ cyhalothrin and biopesticide azadirachtin exposure [59].

GSH is an important non-protein thiol that protects cells against lipid peroxidation [60], [61]. GSH increased gradually on 1 and 7 d at Sheathmar concentrations of 318.6 µg/l and 637.26 µg/l, respectively, in the current study. In the presence of low levels of oxidative stress, this increased level of GSH may act as a protective mechanism against toxicant exposure [62]. On day 14, however, GSH activity decreased significantly at all doses (318.6 µg/l and 637.26 µg/l). This is due to the fact that it uses reduced glutathione to eliminate hydrogen peroxide while also stimulating the production of oxidised glutathione. [62], [63]. GSH is a significant non-protein thiol that protects cells from lipid peroxidation [60], [61]. GSH increased gradually on days 1 and 7 at Sheathmar concentrations of 318.6 µg/l and 637.26 µg/l, respectively. This increased GSH level may be a protective mechanism against toxicant exposure in the presence of low levels of oxidative stress [62]. However, on day 14, GSH activity decreased significantly at all doses (318.6 µg/l and 637.26 µg/l). This is because it uses reduced glutathione to eliminate hydrogen peroxide and stimulate the production of oxidised glutathione [62]- [65]. This significant decrease in GST activity could be attributed to a decrease in GST-related gene expression [66]. Because GST genes are downregulated in response to toxicant exposure, nuclear transcription factors are unable to bind to the relevant promoter region, resulting in increased ROS generation [67].

Similar variations in the GST level were seen in *Tubifex tubifex* treated with profenofos, λ cyhalothrin and azadirachtin [59]. During oxidative stress, ROS interacts with unsaturated fatty acids in membranes, resulting in LPO. Increased LPO levels are associated with increased ROS production [61], [68]. LPO's final product is MDA, a sensitive and delicate oxidative cell damage marker [69]. In the current study, there was a significant increase in MDA activity at all exposure times, as well as an increase in Sheathmar concentrations (318.6 µg/l and 637.26 µg/l) compared to the control ($p<0.05$), indicating increased ROS production [70]. This increase in MDA could be caused by Sheathmar's interaction with polyunsaturated fatty acids, resulting in oxidative stress [64]. Increased MDA levels reduce cell membrane permeability, allowing toxicants to enter the cell and cause DNA damage and, eventually, apoptosis [57]. A similar effect on MDA activity was observed in *Tubifex tubifex* following exposure to profenofos, λ cyhalothrin and azadirachtin e [59].

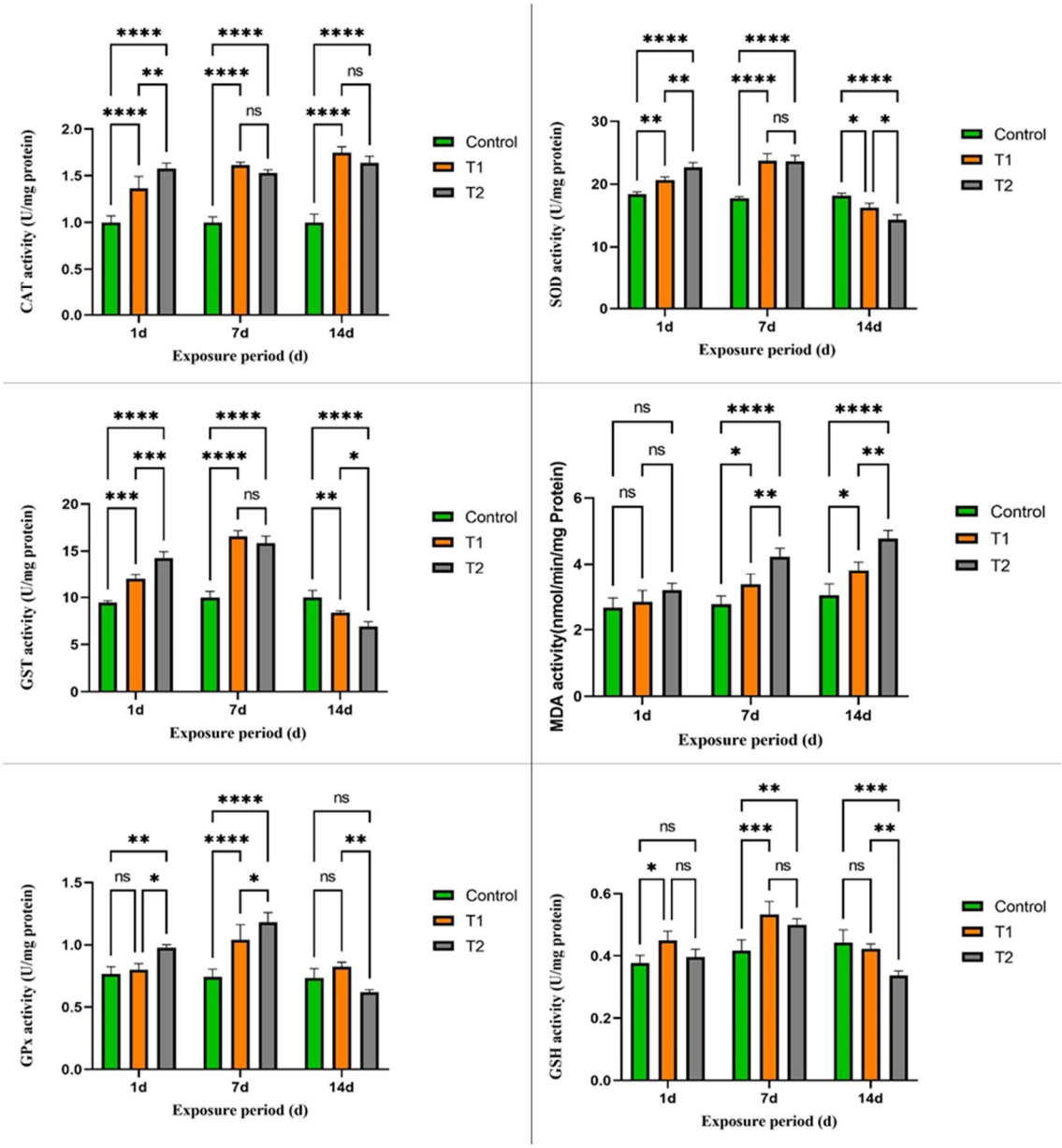


Fig 2: Effects of different sublethal concentrations of Sheathmar on CAT, SOD, GST, MDA GPx and GSH and levels in *Tubifex tubifex* at different exposure periods. T1 and T2 indicate Sheathmar concentration at 10% of 96h LC_{50} value (318.6 µg/l) and 20% of 96h LC50 value (637.26 µg/l).

A correlation matrix plot was created to investigate the overall correlations between pesticides and indicators of oxidative stress. Sheathmar is positively correlated with CAT and MDA, according to the plot.

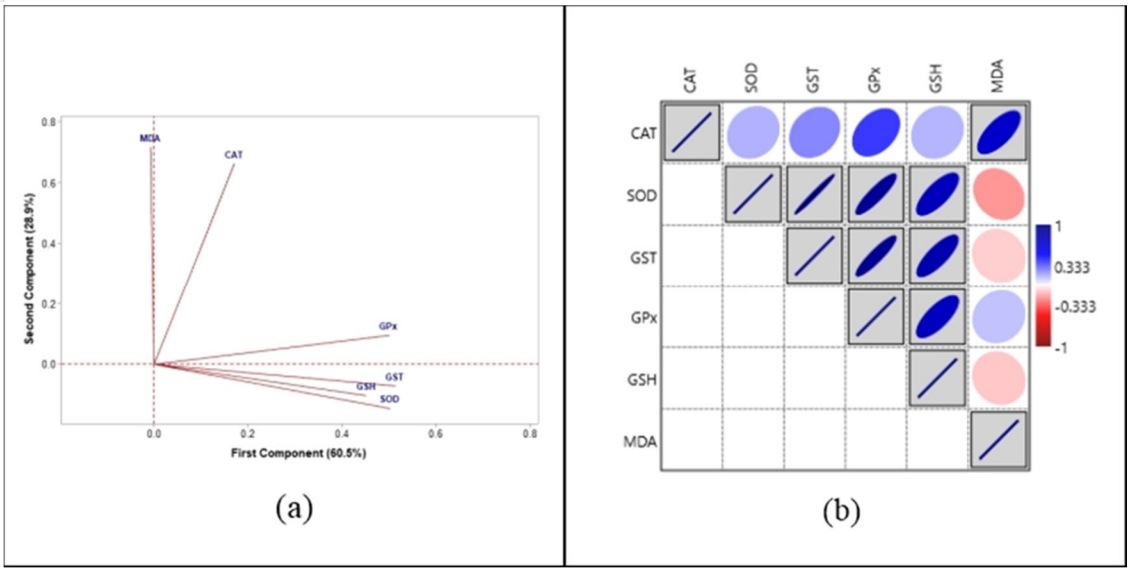


Fig. 3: (a) Ordination diagram of PCA and (b) Pearson correlation matrix plot on stress parameters after 14 days exposure to Sheathmar and oxidative stress biomarkers in *Tubifex tubifex.* Cross indicates p>0.05

The IBR index was used to calculate Sheathmar's overall stress on *Tubifex tubifex*. The IBR conveniently depicts the inclusion of multiple biomarkers in a single value [42]. It is a powerful technique and an effective strategy for assessing the health status of living organisms by coordinating and combining biomarkers [71]. Higher IBR values typically indicate a more distressing ecological condition for the organisms, whereas low IBR scores indicate favourable environmental conditions [72]. The current study shows that T2-7d is the most affected group based on concentration and exposure periods, followed by T2-7d, T1- 7d, T2-1d, T1-14d, T1- 1d, T2-14d, C-7d, C-14d, and C-1d (Fig. 4).

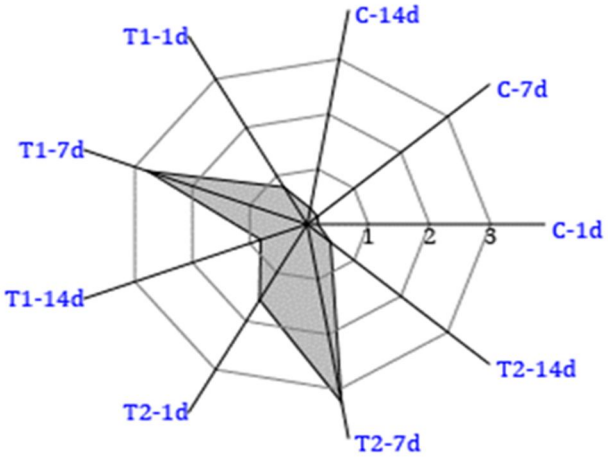


Fig. 4: IBR of oxidative stress parameters measured in *Tubifex tubifex* after chronic exposure to Sheathmar. C indicates control (0 mg/l), T1 indicates Sheathmar concentration at 10% of its 96h LC_{50} value (318.6 µg/l); T2 indicates Sheathmar concentration at 20% of its 96h LC_{50} value (637.26 µg/l).

IV. CONCLUSION

By incorporating the pesticide Sheathmar, *Tubifex tubifex* showed changes in survivability and ethological changes at the acute level, as well as changes in oxidative stress parameters at the sublethal level. As a result, the current study on the toxic effects of Sheathmar against *Tubifex tubifex* implicates that oxidative stress biomarkers are critical attributes for determining the intricate health status of aquatic species. However, more research is needed to extract Sheathmar toxicity on tubificid worms at the ultrastructural level and reduce their toxicity using an adequate plant extract.

V. ETHICAL APPROVAL

This study does not include animal experiments by the authors that require the ethics committee's permission. In particular, no ethical approval is needed for invertebrates such as *Tubifex tubifex.*

VI. FUNDING

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VII. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

VIII. ACKNOWLEDGMENT

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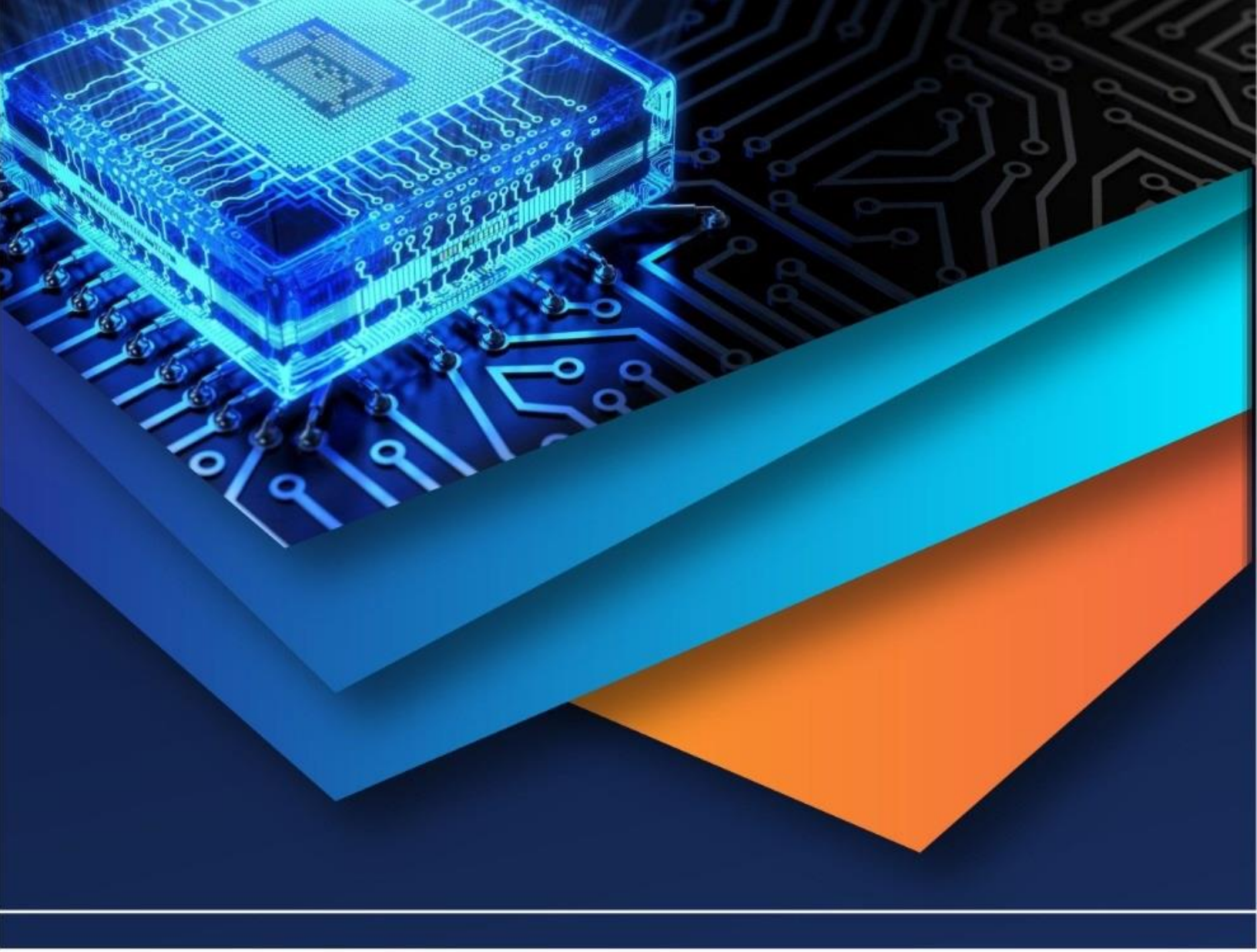

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