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# Acute Toxicity and Sublethal Effects of Lauryl Alcohol Ethoxylate on Oxidative Stress and Antioxidant Defense Parameters in Benthic Oligochaete Worm, *Tubifex Tubifex*

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**Abstract:** The present study aimed to assess the acute toxicity of Lauryl alcohol ethoxylate (LAE) and its sublethal effects on oxidative stress enzymes in *Tubifex tubifex*, a benthic oligochaete worm. The results indicated that the 96-hour median lethal concentration (LC<sub>50</sub>) of LAE is 0.77 mg/l for *Tubifex tubifex*. The model fit performance depicted that GUTS-SD model can better predict the survival rate of *Tubifex tubifex*. Sublethal concentrations of LAE (10% and 20% of the 96h LC<sub>50</sub>) significantly altered the oxidative stress enzymes. Reduced glutathione (GSH), glutathione S-transferase (GST), and glutathione peroxidase (GPx) all displayed a significant initial increase followed by a subsequent decline, whereas catalase (CAT) activity and malondialdehyde (MDA) levels increased significantly at all exposure periods with increasing concentrations of LAE. Moreover, the effects of LAE on *Tubifex tubifex* were demonstrated by the establishment of potency index, integrated biomarker response (IBR) and biomarker response index (BRI) assessment. These findings suggest that exposure of *Tubifex tubifex* to LAE influences the survival of *Tubifex tubifex* at the acute stage and modifies alterations in oxidative stress enzymes at the sublethal level.

**Keywords:** Lauryl alcohol ethoxylate, *Tubifex tubifex*, acute toxicity, oxidative stress, integrated biomarker response, biomarker response index

## I. INTRODUCTION

Surfactants are a wide family of chemical compounds with both hydrophobic and hydrophilic sites required for organic pollutant solubilization. [1]. Surfactants introduced into freshwater can have a substantial impact on the biological system [2]. The total annual use of surfactants is increasing at a steady rate [3]. Although the majority of surfactants are biodegradable, their prolonged use in groundwater and constant dumping on the surface contribute to the aquatic environment's ongoing and repetitive occurrences. [4]. When surfactants cling to macromolecules, they are poisonous to them and interfere with their efficient function in biological systems [5]. Surfactants are toxic to aquatic organisms, according to numerous studies [4], [6]–[8]. There are four types of surfactants: anionic, cationic, non-ionic and zwitterionic. [9]. Out of these, cationic surfactants are compounds that have a lengthy, hydrophobic chain that connects to a positive nitrogen atom. [10]. When compared to anionic surfactants, these are more hazardous and, in particular, are not substituted for several industrial uses [11]. This class of surfactants is widely utilised in a variety of industries, including textiles, emulsifiers, wetting agents, disinfectants, and cosmetics [10], [11]. One such non ionic surfactant with antimicrobial properties is Lauryl Alcohol Ethoxylate [12].

*Tubifex tubifex* is a freshwater sediment-dwelling benthic oligochaete worm. It is a massive species with a global distribution that is robust to a wide range of environmental conditions. It is easily cultivable in laboratories and serves as a valuable food source for fish [13].

While the preliminary toxicity research employs a lethal endpoint such as the LC<sub>50</sub>, sublethal toxicity studies are far more judicious because the species is exposed to significantly lower, biologically relevant hazardous quantities of toxic compounds [14]–[17]. Moreover, the use of general unified survival models (GUTS) has been recommended as a suitable strategy for evaluating toxicant risk in the environment. The damage-related mortality process is defined by two survival strategies: stochastic death (SD) and individual tolerance (IT).

Individuals are comparable in the SD model, and the risk of death from chemical stress increases as damage grows when a specific level of impairment is reached. Individuals, on the other hand, vary in their vulnerability to chemical stress, and once the damage exceeds an individual's threshold, it dies instantly [18], [19].

The metabolism of xenobiotics in organisms significantly contributes to the formation of reactive oxygen species (ROS) [20]. These reactive oxygen species (ROS) effectively start lipid peroxidation (LPO) and cause severe oxidative stress damage to biomolecules like DNA, proteins, and membranes [21].

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, oxidative stress occurs [22]. As a result, an effective and secondary technique for evaluating antioxidant enzyme activity may be relevant in aquatic toxicology studies (Bhattacharya et al., 2021). A few observations addressing oxidative stress alterations in *Tubifex tubifex* following pesticide exposure have been presented [24]–[29]. However, there are few data on the negative effects of surfactants on oxidative stress in these worms [2].

Because single biomarkers cannot give an appropriate and practical assessment of a toxicant's toxicity on aquatic life forms, an amalgamated biomarker analysis is recommended to better understand an organism's reaction to toxic substances [30]. As a result, IBR provides a comprehensive methodology that incorporates all biomarker reactions and plays an important role in determining the toxicity of contaminants [31]. Moreover, BRI has been widely utilized in recent years to integrate multiple biomarker responses. It is rudimentarily focused on the evaluation of the organism's overall health status [32].

As a result, the goal of this study is to assess the acute toxicity of LAE to *Tubifex tubifex* in terms of LC50 values after acute exposure, as well as to investigate the possible toxicity of LAE at sublethal concentrations by monitoring changes in oxidative stress indicators. Then, IBR and BRI are used to determine the toxicity of LAE in *Tubifex tubifex*. The GUTS-SD and IT models were used to assess aquatic species' acute responses to surfactants, anticipate toxicity, and determine which model, SD or IT, best matched the toxicity data.

## II. MATERIALS & METHODS

The appropriate quality assurance procedures for sample processing, storage, and preservation were followed, as specified by the US EPA.

### 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  $25.9 \pm 0.4$  °C, pH  $7.2 \pm 0.6$ , free CO<sub>2</sub>  $16.9 \pm 0.7$  mg/l, dissolved oxygen  $7.1 \pm 0.5$  mg/l). Then, organisms averaging  $11.4 \pm 0.2$  mm in length were added to the experimental setup. The physiochemical characteristics of the test water were maintained during the exposure duration (temperature  $27.2 \pm 0.3$  °C, pH  $7.2 \pm 0.3$ , free CO<sub>2</sub>  $17.8 \pm 0.3$  mg/l, dissolved oxygen  $6.7 \pm 0.5$  mg/l, total alkalinity  $177 \pm 5.2$  mg/l as CaCO<sub>3</sub>, hardness  $120 \pm 4.1$  mg/l as CaCO<sub>3</sub>).

### B. Test Chemicals

The technical grade of LAE was obtained from the chemistry department, The university of Burdwan and other reagents were procured from Sisco Research Laboratories Pvt. Ltd. (SRL), India. The stock solution of LAE (1% w/v) and subsequent dilutions were made following a standard protocol [33].

### C. Bioassay for Acute Toxicity and Survival rate Projection

A static renewal acute toxicity bioassay was carried out in 250 mL glass beakers containing 200 mL water and ten *Tubifex tubifex*. Each experiment was repeated three times. Initially, a range detection test was performed to determine the range of mortality levels. Following that, a final test was conducted by exposing the worms to various nominal concentrations of LAE (0.50, 0.60, 0.70, 0.80, 0.90, 1.00, 1.10, 1.20, 1.30, 1.40, 1.50) for 96 hours, each with a control containing water free of the toxicant. The worms were counted for mortality at 24, 48, 72, and 96 hours. The LC<sub>50</sub> values were determined at 24, 48, 72, and 96 h using Finney's probit analysis, with log concentration as the dependent variable and probit as the independent variable [34]. The survival rate pattern of *Tubifex tubifex* in response to LAE was evaluated using GUTS modeling, which was accomplished using the standalone software OpenGUTS. kd (the dominant rate constant), mw (the median of the threshold distribution), hb (the background hazard rate), and bw (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**

To analyze oxidative stress enzyme parameters at a sublethal level, 2 g of *Tubifex tubifex* is transferred from the stock tank to glass beakers, each holding 1 liter of unchlorinated tap water. Two sublethal concentrations of LAE (10% of 96h LC<sub>50</sub> values, i.e., 0.07 mg/l and 20% of 96h LC<sub>50</sub> values, i.e. 0.15 mg/l) were delivered over periods of 1d, 7d, and 14d. The control worms were placed in another glass beaker with 1l of sterile water free of any toxicant. On day 1, LAE was administered into the experiment (initial treatment). Then, 10% of the test medium was renewed every two days and was replaced with LAE at 10% of the initial nominal concentration. Perpetual aeration was provided during the exposure times. The operation was repeated three times. 1 g of worms were collected and homogenised from each replicate at each exposure period in a 0,1 M phosphate buffer (pH 7.6). Centrifugation at 10000 g for 10 minutes was conducted using a cold centrifuge (Hermle Labortechnik), and the supernatant was kept at -20<sup>0</sup> C until further analysis. The protein content was evaluated using the Bradford technique [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). In contrast, MDA levels were quantified in nanomoles of thiobarbituric acid reactive substance (TBARS) per minute per milligram of protein (nmol TBARS/min/mg protein).

**E. Determination of IBR and BRI**

The data on oxidative stress biomarkers were articulated utilizing an IBR system based on the protocol of Beliaeff and Burgeot (2002) and expressed in radar plots. Moreover, the biomarker response index (BRI) for determining the health status of the organism using standard protocol [32]

**F. Statistical Analysis**

The LC<sub>50</sub> values were calculated using Finney's probit analysis in Microsoft Excel 2013. Survival curves were established using Kaplan-Meier analysis. A two-way ANOVA followed by the Tukey post hoc test was used to identify the comparisons between controls and exposed worms. The analyses are summarised as mean ± standard deviation. Mean values with a p<0.05 significance level is considered statistically significant. A correlation matrix plot was used to determine the associations between oxidative stress indicators.

**III. RESULT AND DISCUSSION**

The LC<sub>50</sub> values of LAE to *Tubifex tubifex* associated with 95% confidence intervals are depicted in Table 1 and are reported to be 1.00, 0.93, 0.87 and 0.77 mg/l, respectively. Hence based on the LC<sub>50</sub> values, LAE is considered moderately toxic to *Tubifex tubifex*

Table 1: The LC<sub>50</sub> values and 95% confidence limits of LAE to *Tubifex tubifex* at different exposure periods (24, 48, 72 and 96 h).

Exposure period (h)	LC <sub>50</sub> ± SE (mg/l)	95% confidence limit	
		Lower	Upper
24	1.00 ± 0.020	0.920	1.099
48	0.93 ± 0.021	0.852	1.032
72	0.87 ± 0.030	0.767	1.003
96	0.77 ± 0.025	0.688	0.861

Moreover, the survivability curve also depicts that LAE significantly affected the overall survival rates of *Tubifex tubifex* in a dose and duration-dependent manner with respect to control (Mantel log-rank test; p < 0.05) (Fig 1). The 100 % survivability of *Tubifex tubifex* is observed in control at all exposure periods (24, 48, 72 and 96 h).

However, with the increase of concentration of LAE as well as periods of exposure (24, 48, 72 and 96 h), the survivability rate of *Tubifex tubifex* decremented significantly (Mantel log-rank test; p < 0.05).

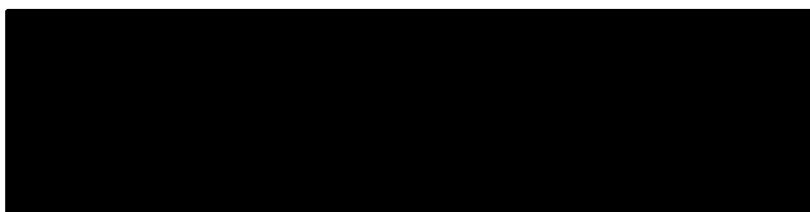
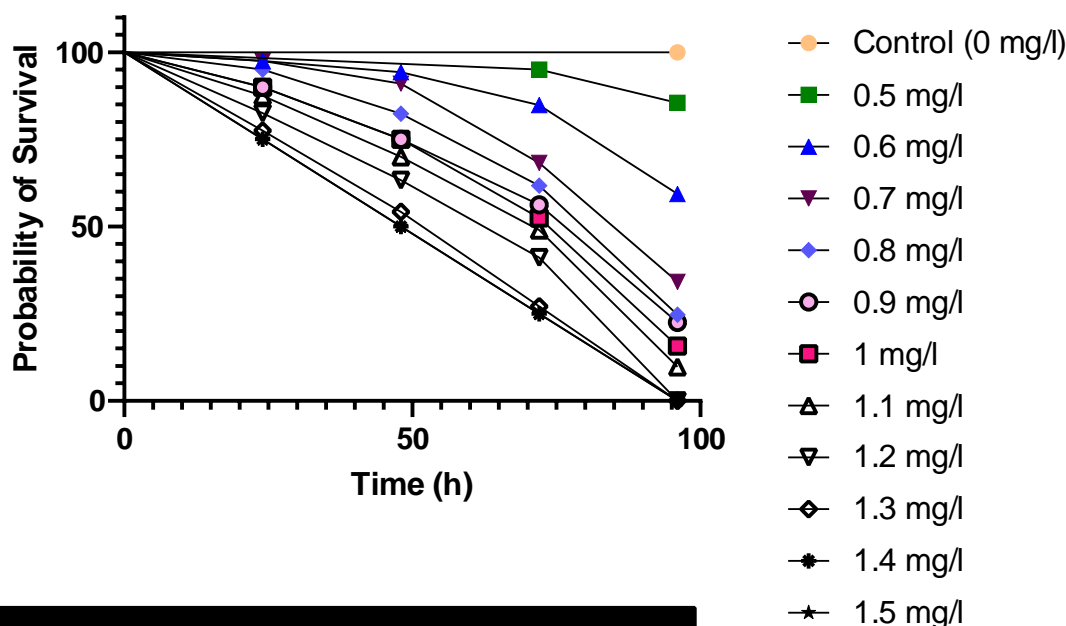


Fig 1: Kaplan-Meier survival curves of *Tubifex tubifex* exposed to different concentrations of LAE (0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 and 1.5 mg/l) at different exposure periods (24, 48, 72 and 96 h).

The model parameters and the fitted performance of GUTS (SD or IT) are given in Table 2. The fitted performance of GUTS-IT was better than that of GUTS-SD in the case of LAE based on AIC values (a smaller AIC value indicates the best fit). Thus, the model simulation illustrated that the GUTS-IT model could better predict the survival rate observed in *Tubifex tubifex* for surfactant exposure than the GUTS-SD model at an acute level. The survival model output demonstrates that the model deducing SD or IT should be chosen wisely to determine the toxic effects of various toxicant exposure patterns. It is clear that such mechanistic modeling has significant potential for enhancing the accuracy of environmental risk management in the future and can significantly help in effective decision-making. Based on the GUTS-IT model, the 100d LC<sub>50</sub> value was determined and depicted in Table 3.

Table 2: Model parameters in case of LAE [K<sub>d</sub> indicates Dominant rate constant; m<sub>w</sub> indicates Threshold for mortality; b<sub>w</sub> indicates Killing rate; h<sub>0</sub> indicates background hazard rate & F<sub>s</sub> indicates Spread factor of the threshold distribution]

Symbol	GUTS-RED		unit	AIC Value	
	SD	IT		SD	IT
k <sub>d</sub>	143.8 (0.001641 - 143.8)	1.075 (0.001641 - 143.8)	d <sup>-1</sup>	<b>247.18</b>	251.13
m <sub>w</sub>	0.4856 (0.001368 - 1.485)	0.701 (0.001368 - 3)	mg/l		

$b_w$	1.04 (0.01756 – 222967)	-	L/mg/d
$h_b$	$1e^{-6}$	$1e^{-6}$	$d^{-1}$
$F_s$	1	2.269 (1.05 – 20)	

Moreover, the forecasted  $LC_{50}$  values from GUTS-SD models are given in Table 3

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

Time [d]	$LC_{50}$ GUTS-SD (mg/l)
1	1.159 (1.024 – 1.356)
2	0.821 (0.7537 – 0.9142)
3	0.7088 (0.6593 – 0.7705)
4	0.6529 (0.6089 – 0.6992)
7	0.5811 (0.5364 – 0.6103)
14	0.5333 (0.4836 – 0.5526)
100	0.4923 (0.437 – 0.5058)

Antioxidant enzymes are direct biomarkers of oxidative stress, capable of neutralizing reactive oxygen species (ROS) and other pro-oxidative enzymes in cells under typical conditions [43], [44]. The effect of LAE on different antioxidant enzymes is depicted in Fig. 2 CAT is a critical enzyme that effectively neutralizes reactive oxygen species (ROS) in the antioxidant system and degrades  $H_2O_2$  to molecular oxygen and water [45], [46]. Catalase activity increased significantly at 0.07 mg/l and 0.15 mg/l of LAE during the 1, 7 and 14 d exposure period compared to the control group ( $p < 0.05$ ). This increase in CAT results in increased nuclear Nrf2 expression, which protects cells from  $H_2O_2$ -induced stress [47]. Mosleh et al. (2014) observed a uniform increase in CAT activity in *Tubifex tubifex* following exposure to pyrimethinal fungicides.

SOD is the most important oxidative stress enzyme because it provides significant resistance against oxidative stress by converting reactive oxygen radicals to hydrogen peroxide [48]–[51]. SOD activity increased significantly at 0.07 mg/l and 0.15 mg/l of LAE during the 1 and 7 d exposure period but decreased considerably at 0.07 mg/l and 0.15 mg/l of LAE during 14 d exposure period in comparison to the control group ( $p < 0.05$ ). This increase in SOD activity could be related to the stimulation of superoxide ions, which activate the formation of SOD, which protects cells from oxidative damage [52]. On day 14, however, the decrease in SOD activity is probably related to the excessive formation of ROS as a result of toxic pollution, which harmed or inactivated SOD's action by oxidizing the cysteine in SOD or by reducing the expression of SOD-related genes [53].

GPx alleviates possible oxidative stress by accelerating the conversion of hydrogen peroxide to water and oxygen. When GPx is blocked, more hydrogen peroxide is accessible, resulting in tissue degradation and oxidative stress. GPx activity is always specifically linked to GSH concentration. This is because it promotes the synthesis of oxidized glutathione by utilizing reduced glutathione to remove hydrogen peroxide [54]. In the present study, GPx activity increased significantly on 1 and 7 d at all LAE concentrations (0.07 mg/l and 0.15 mg/l, respectively) and appeared to play a significant role in antioxidant protection [55]. At the end of the 14-day exposure period, GPx activity reduced significantly compared to the control level at all LAE concentrations (0.07 mg/l and 0.15 mg/l, respectively) ( $p < 0.05$ ). This decrease in GPx could result from the antioxidant defense system's failure to inhibit toxicant-induced ROS generation [56]. This decrease may indicate that the amount of hydroperoxide formed during lipid peroxidation exceeds the antioxidant capability [57]. Additionally, these changes in GPx activity are most likely due to changes in GPx mRNA expression [58]. Similar results were observed in *Tubifex tubifex* following a 15-day thallium exposure (Kiliç and Kiliç, 2017).

GSH is a significant non-protein thiol that protects cells from lipid peroxidation [60], [61]. In the current study, GSH increased gradually on 1 and 7 d at LAE concentrations of 0.07 mg/l and 0.15 mg/l. This increased level of GSH 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 considerably at all doses (0.07 mg/l and 0.15 mg/l). This is because it utilizes reduced glutathione to eliminate hydrogen peroxide and stimulate the production of oxidized glutathione. [62], [63]. This GSH depletion is most likely due to glutathione peroxidase oxidation in response to increased free radicals. GSH levels may fluctuate due to sudden variations in glycolysis rate and Krebs cycle activity, resulting in mitochondrial malfunction [64].

GST is a critical biotransformation enzyme in the phase II phase that regulates the accumulation of GSH and xenobiotics and is widely regarded as a crucial component of the detoxification mechanism [65]. GST activity was significantly increased on 1 and 7 d when *Tubifex tubifex* was subjected to different dosages of LAE (0.07 mg/l and 0.15 mg/l) ( $p < 0.05$ ). This increase in GST activity could result from a faster rate of glutathione disulfide (GSSG) synthesis (Li et al., 2010). However, a significant decrease in GST activity in *Tubifex tubifex* at different LAE doses (0.07 mg/l and 0.15 mg/l) during the 14d of exposure time as compared to control ( $p < 0.05$ ) indicated that the worm's detoxification mechanism was impaired under long-term exposure [62]. This considerable decrease in GST activity could be due to the downregulation of GST-related gene expression [66]. Due to the downregulation of GST genes in response to toxicant exposure, nuclear transcription factors cannot 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 chitosan [25].

ROS interacts with unsaturated fatty acids in membranes during oxidative stress, resulting in LPO. Increased LPO levels imply an increase in ROS production [61], [68]. MDA, a sensitive and delicate oxidative cell damage marker, is the culminating product of LPO [69]. In the present study, a substantial increase in MDA activity at all exposure periods was observed, along with an increase in LAE concentrations (0.07 mg/l and 0.15 mg/l) as compared to control ( $p < 0.05$ ), indicating increased ROS production [70]. This increase in MDA could result from the interaction of LAE with polyunsaturated fatty acids resulting in oxidative stress [64]. Increased MDA levels affect the permeability of the cell membrane, allowing toxicants to enter the cell, resulting in DNA damage and eventually apoptosis [57]. A similar effect on MDA activity was observed in *Tubifex tubifex* following 15d exposure to thallium [59]

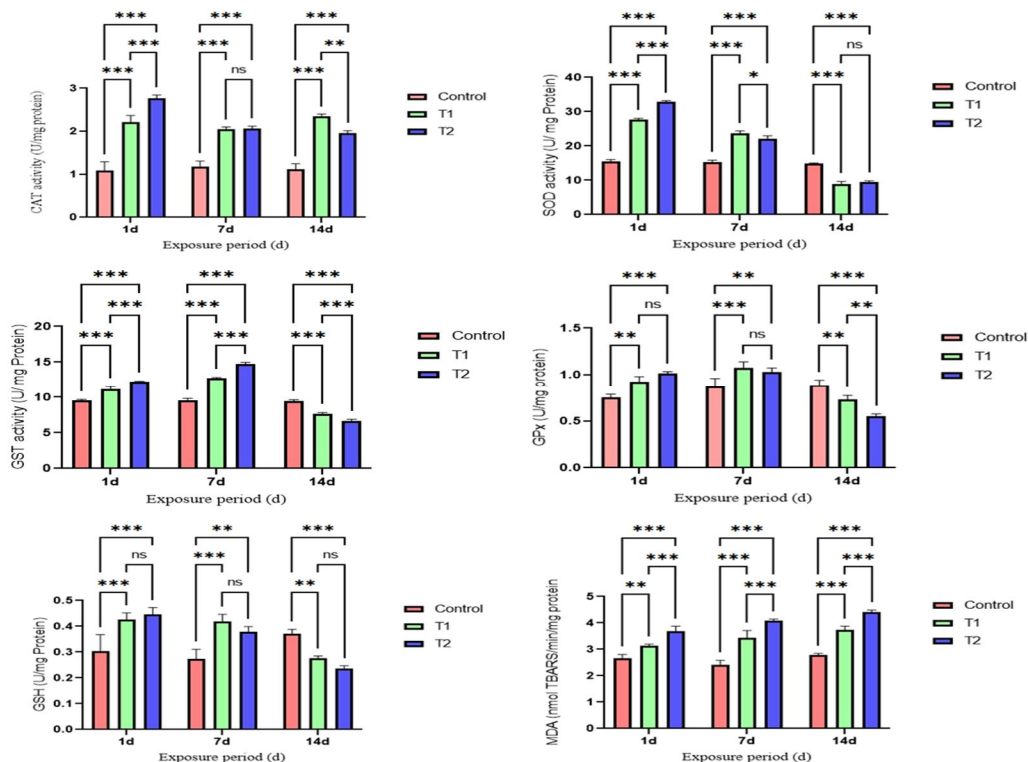


Fig 2: Effects of different sublethal concentrations of LAE on a) CAT, b) SOD, c) GPx, d) GSH, e) GST and f) MDA levels in *Tubifex tubifex* at different exposure periods. Different letters (a-c) indicate significant difference within the same exposure period ( $p < 0.05$ ). T1 and T2 indicate LAE concentration at 10% of 96h LC<sub>50</sub> value (0.07 mg/l) and 20% of 96h LC<sub>50</sub> value (0.15 mg/l).

Two-way ANOVA observation depicted that LAE concentration, time of exposure, and their interactions vigorously impacted all of the oxidative stress biomarkers being investigated (CAT, SOD, GST, GPx, GSH, and MDA or LPO) ( $p < 0.05$ ) (Table 4).

Table 4: Two-way ANOVA for LAE concentration in mg/l (LAE) and period of exposure in days (exposure period) on oxidative stress biomarkers in *Tubifex tubifex* after sublethal exposure to LAE.

Source	Sum of Squares	DF	F	Sig.
CAT				
Exposure period	7.349	2	F (2, 18) = 287.4	P<0.001
LAE	0.3387	2	F (2, 18) = 13.24	P<0.001
Exposure period x LAE	0.9683	4	F (4, 18) = 18.93	P<0.001
SOD				
Exposure period	193.4	2	F (2, 18) = 276.9	P<0.001
LAE	927.5	2	F (2, 18) = 1328	P<0.001
Exposure period x LAE	467.2	4	F (4, 18) = 334.4	P<0.001
GST				
Exposure period	13.28	2	F (2, 18) = 175.2	P<0.001
LAE	91.99	2	F (2, 18) = 1214	P<0.001
Exposure period x LAE	51.50	4	F (4, 18) = 339.8	P<0.001
GPx				
Exposure period	0.02450	2	F (2, 18) = 4.576	P=0.025
LAE	0.3437	2	F (2, 18) = 64.18	P<0.001
Exposure period x LAE	0.3235	4	F (4, 18) = 30.21	P<0.001
GSH				
Exposure period	0.01588	2	F (2, 18) = 8.268	P=0.003
LAE	0.04395	2	F (2, 18) = 22.88	P<0.001
Exposure period x LAE	0.07108	4	F (4, 18) = 21.48	P<0.001
MDA				
Exposure period	9.513	2	F (2, 18) = 215.5	P<0.001
LAE	1.100	2	F (2, 18) = 24.93	P<0.001
Exposure period x LAE	0.4526	4	F (4, 18) = 5.126	P=0.006



To investigate the overall correlations between surfactants and indicators of oxidative stress, a correlation matrix plot was constructed. According to the plot LAE is positively correlated with CAT and MDA.

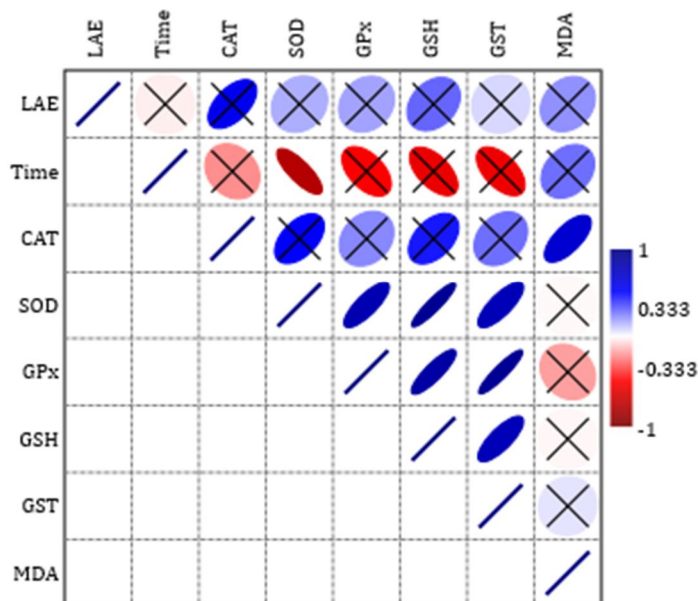


Fig. 3: Pearson correlation matrix between LAE concentration (mg/l), exposure period (d) and oxidative stress biomarkers in *Tubifex tubifex*. Cross indicates  $p > 0.05$

The IBR index was applied to quantify the overall stress of LAE on *Tubifex tubifex*. The IBR portrays the inclusion of several biomarkers in a single value conveniently [42]. It is a puissant technique and an efficacious strategy to evaluate the health status of living organisms by coordinating and amalgamating biomarkers [71]. Higher IBR values commonly indicate the more distressing ecological condition for the organisms, whereas low scores of IBR demonstrate favorable environmental conditions [72]. Based on concentration and exposure periods, the present study shows that T2-14d is the highest affected group, followed by T1-14d, T2- 1d, T1-1d, T2-7d, C-1d, C-7d, T1-7d and C-14d (Fig. 4). In integration, biomarker weights and scores for exposed worm parameters are used to calculate BRI, which conventionally reflects the general health condition of worm [32]. The BRI values of LAE are within 0-2.6 (Fig. 5), which portrays paramount alterations from the normal [32]. Thus, it is conspicuous from our finding that LAE adversely impacts worm health.

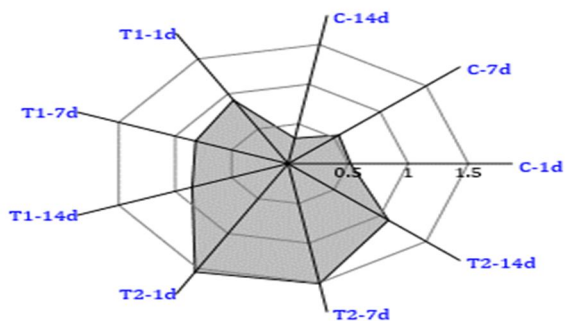


Fig. 4: IBR of oxidative stress parameters measured in *Tubifex tubifex* after chronic exposure to LAE. C indicates control (0 mg/l), T1 indicates LAE concentration at 10% of its 96h LC<sub>50</sub> value (0.07 mg/l); T2 indicates LAE concentration at 20% of its 96h LC<sub>50</sub> value (0.15 mg/l).

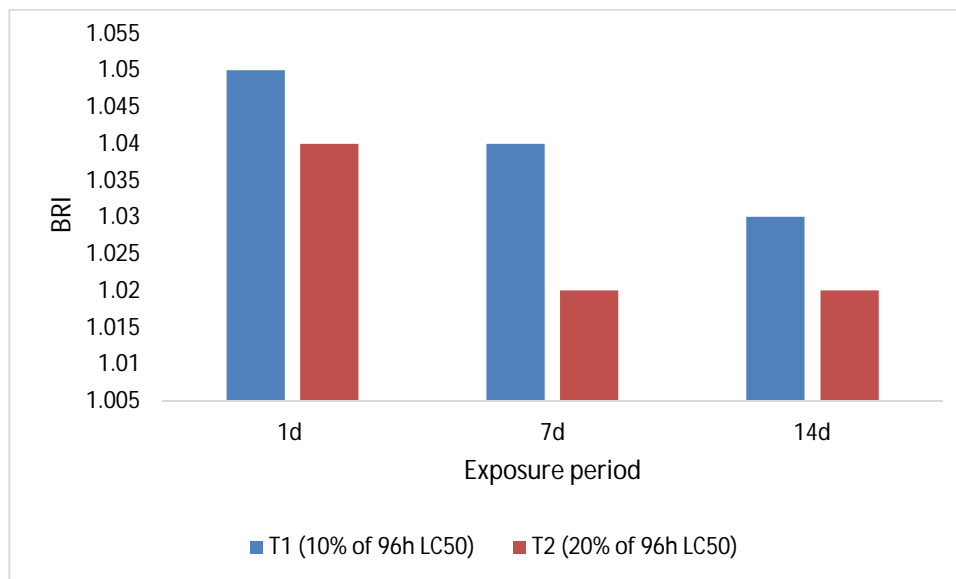


Fig. 5: BRI values representing the health status of *Tubifex tubifex*. upon exposure to LAE

#### IV. CONCLUSION

The finding of this study revealed that *Tubifex tubifex* showed alterations in survivability and ethological changes at the acute level and modifications in oxidative stress parameters at the sublethal level by incorporating surfactant LAE. Consequently, the present study on the toxic effects of LAE against *Tubifex tubifex* implicatively indicates that oxidative stress biomarkers are the critical attributes for ascertaining aquatic species' intricate health status. However, further studies are needed to extract LAE toxicity on tubificid worms at the ultrastructural level and to reduce their toxicity by utilizing adequate plant extract.

##### A. 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*.

##### B. Funding

The research did not receive any specific grant from funding agencies in public, commercial or nonprofit sectors.

##### C. Conflict of Interest

The authors declare that they have no conflict of interest.

#### V. ACKNOWLEDGMENT

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