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ARTICLE

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FORMIC ACID INDUCED ACUTE TOXICITY AND ITS SUBLETHAL EFFECTS ON GROWTH, BEHAVIORAL PATTERN AND OXIDATIVE STRESS PARAMETERS OF THE FRESHWATER SNAIL *BELLAMYA BENGALENSIS*

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The organic acid, namely formic acid is discharged into the water bodies from various paper, leather tanning, and textile processing industries causing a potential threat to the aquatic life forms. This study evaluated the acute and sublethal toxicity of formic acid by assessing the mortality, behavioral alterations, and changes in the levels of oxidative stress enzymes in the freshwater snail, Bellamya bengalensis. The acute toxicity (96h LC50) value of formic acid to B. bengalensis is 182.69 mg/l. Various behavioral alterations like crawling movement, tentacle movement, touch reflex, and mucous secretion were also noted among treated and controlled snails. The effect of sublethal concentration of formic acid on differential expression of oxidative stress enzymes was investigated. Integrated biomarker response (IBR) and biomarker response index (BRI) analysis illustrate an overall summative representation of oxidative stress parameters and the health status of B. bengalensis. Moreover, toxicokinetic-toxicodynamic and species sensitivity distributions studies performed in the study will be helpful for ecological risk management. Therefore, the study concludes that exposure to formic acid affects survivability and behavior by generating oxidative stress in B. bengalensis.

Highlights

- Formic acid pollution in the aquatic ecosystem causes an immense threat to *Bellamiya bengalensis*.
- Chronic exposure to this acid results in behavioral alteration, oxidative stress and death of the animals.
- IBR and BRI analyses were performed for ecological risk assessment and to determine the organism's health condition.
- Prediction of 100 days LC50 value was performed through GUTS modeling.

Introduction

Water is the primary natural resource, without which life forms fail to exist. Due to rapid industrialization, the exploitation of natural water bodies has increased substantially resulting in water pollution. The major sources of water pollution include industrial effluents and agricultural run-off. Agricultural lands drain organic chemicals, pesticides, drugs, etc. into the water bodies thereby contaminating the water. Various industries such as pharmaceuticals, textiles, leather, food

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processing, pulp and paper industries generate volumetric waste including toxic chemicals, heavy metals, oil, organic acids, dyes, hydrocarbons, detergents etc. and disposed of in the water bodies[1-4]. In addition, municipal wastewater in untreated conditions, released into the nearby water bodies contains toxic chemicals, solvents and heavy metals[5]. These toxic chemicals cause various degrees of health hazards to the aquatic organism and humans, according to their chemical nature and persistence in the food chain[6].

Formic acid is a very common organic acid frequently used in several industries like pharmaceutical, rubber, textile, pulp and paper etc. and released into the natural water bodies. It is principally used as a preservative and antimicrobial agent in livestock feed. Formic acid is used to preserve the winter feed of cattle. When sprayed over the fresh hay, it prevents the decay process and maintains the nutritive value. In poultry farming, this organic acid is added to limit *Salmonella sp*.[7]. Formic acid is used in industries to prepare drugs, dyes, insecticides and refrigerants[8]. In the rubber industry, formic acid is used as a latex coagulant to get high-quality rubber[9]. In the pulp and paper industry, formic acid is used in pulping process of rice straw to manufacture cellulose fiber with silica[10]. Formic acid is also used in the leather and textile processing industry, as an efficient pH regulator[11].

As formic acid is released to the water bodies from various sectors so, aquatic animals are directly exposed to this chemical. Humans are also exposed to this toxic chemical by consuming these aquatic organisms as food material. Formic acid at high concentration generates strong fumes which cause damage to the eyes, skin and mucosal surface of the mouth and respiratory tract[8]. Inhalation of this toxic chemical causes cough, bronchitis, inflammation in the mucus membrane and breathing discomfort. Ingestion of formic acid causes necrosis in the mucus membrane of the buccal cavity, throat, esophagus and stomach. Extensive exposure to formic acid can causes depression in the central nervous system, metabolic disorder and deterioration in kidney function[12]. Chronic exposure to formic acid results in hematuria and albuminuria. In addition, formic acid is also detected as a toxic intermediate in methanol poisoning[8].

Molluscan species are critically important to monitor water pollution, as they are very important in the food chain and also found in wide geographical areas[13]. Therefore, many molluscan species have already been considered as a good bioindicators measuring water contamination[14]. These animals act as a secondary consumer in the freshwater ecosystem consuming the planktons, aquatic vegetation, worms, animal wastes and other snails also. They are sedentary and have a good filter-feeding mechanism during their food intake. No eco-toxicological research of organic acid toxicity has been conducted previously on snail species and the effect of this toxic substance on a snail is also not clear. Taking into account, this present study aims to investigate the formic acid toxicity of freshwater snail species, *Bellamya bengalensis*, which is used as cheap food source in West Bengal, India. This study includes the acute toxicity and behavioral alterations of these animals in toxicant exposure. In chronic exposure, the oxidative stress biomarkers were also monitored. Furthermore, integrated biomarker response (IBR) was generated for better understanding of formic acid toxicity and biomarker response index (BRI) was used to assess the potential health status in toxic environment. The GUTS modelling was performed for environmental risk assessment of this toxicant exposure. Therefore, it is anticipated that this study will be helpful to access water pollution level due to formic acid toxicity and to establish the safe limit.

Materials and Methods

Experimental Organism: Healthy and active specimens of *Bellamya bengalensis* (Phylum: Mollusca, Class: Gastropoda, Order: Architaenioglossa, Family: Viviparidae) with an average body weight 2.92 ± 0.27 g and shell height 2.35 ± 0.41 cm were collected from the local market of Burdwan, West Bengal, India. Before setting the experiment, the snails were acclimatized in laboratory condition for 1 week with unchlorinated tap water at room temperature (temperature 27.5 ± 0.45 ^{o}C) and under a continuous aerated system. The water was replaced every 24 h to avoid any detritus load. During this period, the health condition of the animals was observed. Only healthy individuals were selected and transferred for further experiment.

Test Chemicals: The analytical grade of formic acid and the reagents used for oxidative stress analysis were procured from Sisco Research Laboratories Pvt. Ltd. (SRL), India.

Acute Toxicity (static renewal) Bioassay: To determine the LC50 value of formic acid, 96 hours acute

toxicity test was conducted in laboratory conditions by exposing *B. bengalensis* (n=10) to different concentrations of this organic acid. This experiment was performed in 15 l glass aquaria each containing 10 l of water. Initially, a range-finding test was performed to estimate the range of concentration of formic acid, where mortality of the animal occurs. During the period of acute toxicity bioassay, 10% of the test medium was substituted at every 24 h and the desired quantity of the organic acids was added immediately to maintain a constant concentration of the toxicant in the solution. The mortality of the snails was recorded at each of four-time intervals: 24, 48, 72 and 96 h. The mortality of the animals was confirmed once they do not show any operculum movement while touching. The dead animals were removed immediately from the experimental setup to avoid any organic decomposition and oxygen depletion. The experiment was performed thrice to achieve a greater statistical significance. Then median lethal concentration (LC50) for 24, 48, 72, and 96 h (confidence limits 95%) of formic acid to *B. bengalensis* was determined using statistical software, Finney Probit program[1,15]. The values of percent mortality of the snails were subjected to statistical tests with the help of SPSS16 and Grpahpad prism to determine the significant variations among the mean mortality of test animals at different concentrations of the organic acid.

Study of the Behavioral Alterations: Behavioral alterations in the snails like crawling movement (CM), tentacle movement (TM), touch reflex (TR) and mucous secretion (MS) were observed and recorded by exposing to different concentrations of the organic acid during the experimental period. A semi-quantitative scoring technique was used to express the behavioral alterations that occurred during their 96 h exposure period to the toxicant[16].

Study of Oxidative Stress Biomarkers: Oxidative stress enzyme parameters were analyzed from chronic toxicity tests by exposing the snails to formic acid for 28 days period. Chronic toxicity tests were conducted in 15 l glass aquaria each containing 10 l of water and 15 healthy and active snail samples. The snails were fed some aquatic vegetation during the experimental period. Two sublethal concentrations of the toxicant i.e. 10% and 20% values of 96 h LC50 value to *B. bengalensis* (18.2 mg/l and 36.4 mg/l) were used during chronic toxicity bioassay. 10% of the test medium was replaced every week during the experiment. The hepatopancreas of the snails from control and treated groups of each replicate were collected at every 7 d intervals with a total exposure period of 28 d and the organs were dried with filter paper to avoid extra water content. The samples were then homogenized in phosphate buffer (0.1 M, pH 7.6) and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was collected and stored at -20ºC for enzyme extract. Bradford standard protocol was used to measure the protein contents of each sample using BSA (bovine serum albumin) as a standard. Superoxide dismutase (SOD) activity was estimated by detecting the inhibition of the photoreduction of nitroblue tetrazolium (NBT)[17]. Catalase (CAT) activity was measured by determining the absorption of residual H_2O_2 spectrophotometrically at 240 nm using the standard protocol[18]. The level of malondialdehyde (MDA) was assayed by estimating the formation of thiobarbituric acid-reactive substances (TBARS) using the standard method of Ohkawa[19]. The SOD and CAT activity was expressed as the unit of U/mg protein while the MDA level was expressed as nmol TBARS/min/mg protein. All the enzyme activity was measured using the UV-visible spectrophotometer (Cecil Aquarius CE 7400).

Development of Integrated Biomarker Response (IBR) and Biomarker Response Index (BRI): The integrated biomarker response (IBR) was used to summarize a battery of oxidative stress biomarkers into a single index[20]. IBR is an effective tool for ecological risk assessment to predict the effect of toxic substances in the environment and to simply interpret the relationship between multiple biomarkers and their toxicity levels[21]. To assess the response of *B. bengalensis* exposed to formic acid, selected oxidative stress biomarker data were integrated through IBR using the standard protocol with minor modifications and represented through star plot[20].

A biomarker response index (BRI) was used to assess the potential health status of the snails during toxicant exposure. The BRI was developed based on the standard protocol described by Hagger [22].

Generation of Species Sensitivity Sistributions (SSD): To generate the species sensitivity distributions **(**SSD), a group of aquatic species was identified and the LC50 value on their formic acid exposure was collected[23]. According to the concentration of formic acid, the aquatic species was ranked from low to high. The ranks were then transformed into proportions by using the following equation: proportion = (rank – 0.5)/number of species. A linearized log-normal curve was drawn by plotting organic

acid concentration on the X-axis and the proportion of species affected on the y-axis[24]. Further, proportions were converted to probit values, which were an inverse cumulative distribution function of the normal distribution with a mean of 5 and an SD of 1. A mean of 5 was selected to make sure that all probit values were non-negative. A central tendency was obtained by regressing log10 concentrations (x-axis)* probit (y-axis)[24].

Survival Rate Analysis: The GUTS modeling (employing Open GUTS standalone software) was used to predict the survivability rate of *B. bengalensis* under chronic exposure to formic acid. The output results were authenticated by juxtaposing the obtained survival rate with the experimental survival rate on 96 h acute toxicity test.

Statistical Analysis: The result so derived is represented statistically harnessing the Graphpad prism and MS-Excel 2016. The results were outlined as mean ± standard deviation (SD). The permissible level of statistical significance was $p < 0.05$.

Results

Acute Toxicity: To measure the lethal concentration of 50% or LC50 values of formic acid on *Bellamya bengalensis*, an acute toxicity test was conducted. The LC50 values were calculated from the number of mortalities observed in presence of various concentrations of the organic acid. The 24, 48, 72 and 96 h LC50 values of formic acid on *B. bengalensis* were determined as 207.32, 199.22, 191.73, 182.69 mg/l respectively (Table 1). The Kaplan Meier survivorship plot was demonstrated to represent the concentration and duration-dependent adverse effect of the survival rate of *B. bengalensis* compared to the control group (Fig. 1). No mortality was recorded in the control group of snails until the 96 h exposure period (Fig. 1). The survival plot shows a significant decrease ($p < 0.05$) in the survival rate of formic acid exposed snails in a concentration and duration-dependent manner. Moreover, 2-way ANOVA also showed that mortality of snails was significantly affected by exposure concentration of toxicant ($p < 0.0001$) and exposure period ($p < 0.0001$).

Table 1 : Median lethal concentration (LC50) values of formic acid in *Bellamya bengalensis*.

Exposure period (h)	LC50 value (mg/l)	95 % confidence limit (mg/l)
24	207.323	198.753-216.262
48	199.221	191.506-207.247
72	191.738	183.871-199.941
96	182.690	174.841-190.892

Behavioral Changes: The behavioral alterations observed in the snails exposed to different concentrations of formic acid are presented in Table 2. The snails of the control group were active and showed normal behavioral patterns throughout the experimental period. However, the experimental groups expressed some abnormal behavior in their crawling movement (CM), tentacle movement (TM), touch reflex (TR) and mucous secretion (MS). The snails in the experimental groups showed normal crawling movement initially but gradually decreased with the increasing concentration of the toxicant and the exposure time. On another hand, more tentacle movement was observed in the experimental snail groups with the increasing concentration of organic acid which finally ceased in a higher dose of the organic acid. The touch reflex was reduced in the formic acid exposed snail groups

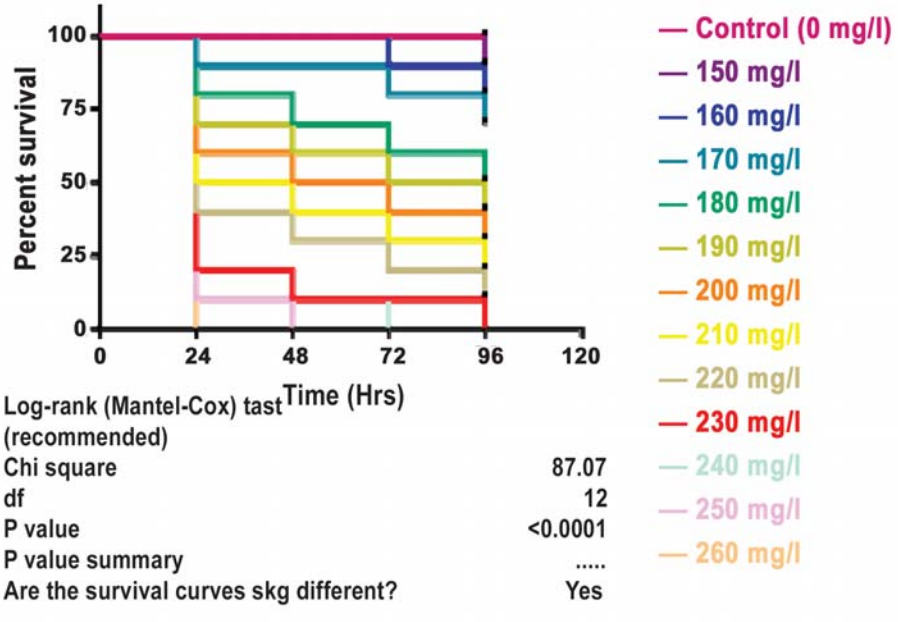


Figure 1. Kaplan-Meier survival plot of *Bellamiya bengalensis* upon exposure to different concentrations of formic acid (0-260 mg/l). X-axis depicting time in hours and Y-axis depicting percent survival.

Table 2 : Changes in the behavioural pattern of *Bellamya bengalensis* after exposure to various concentrations of formic acid at different exposure period.

(Crawling movement (CM), tentacle movement (TM), touch reflex (TR), mucous secretion (MS), –: absent, +: mild, ++: moderate, +++: high)

Conc. mg/l	CM				TM				TR				MS			
	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
0	+++	+++	+++	+++	-	-	-	-	+++	+++	+++	+++	-	-	-	-
150	+++	+++	+++	+++	-	-	-	-	+++	+++	+++	+++	-	-	-	-
160	+++	+++	+++	++	-	-	-	+	+++	+++	++	++	-	-	-	-
170	+++	+++	++	++	-	-	++	++	+++	++	++	+	-	-	-	-
180	+++	+++	++	+	-	+	++	++	+++	++	+	+	-	-	-	+
190	+++	++	++	+	-	+	++	+++	++	+	+	+	-	-	+	+
200	+++	++	+	-	+	++	++	+++	++	+	+	-	-	+	++	++
210	++	++	+	-	+	++	++	++	++	+	+	-	-	+	++	++
220	++	++	+	-	+	++	++	+	++	+	-	-	+	+	++	+++
230	++	+	-	-	+	+	+	-	+	-	-	-	++	++	++	+++
240	+	+	-	-	-	-	-	-	-	-	-	-	++	++	+++	+++
250	+	-	-	-	-	-	-	-	-	-	-	-	++	+++	+++	+++
260	-	-	-	-	-	-	-	-	-	-	-	-	++	+++	+++	+++

with the increasing concentration of toxicant compared to the control group and nearly no response was recorded at 96 h exposure period. The snails, exposed to the toxicant expressed extensive mucous secretion with increasing concentration of toxicant and exposure time.

Biomarkers of Oxidative Stress: The normal cellular environment has an antioxidant defense system for scavenging the free radicals generated during the metabolic processes[25]. However, various environmental stressors can induce oxidative damage in cells due to excessive production of reactive oxygen species (ROS), which interfere with the balance between oxidative stress and antioxidant defense mechanism[26]. Antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) level were significantly increased ($p < 0.0001$) in *B. bengalensis* after chronic exposure to two sublethal concentrations of formic acid (10% and 20% of the LC50 values) compared to the control group (Fig. 2A, B). In addition to the alteration of the antioxidant

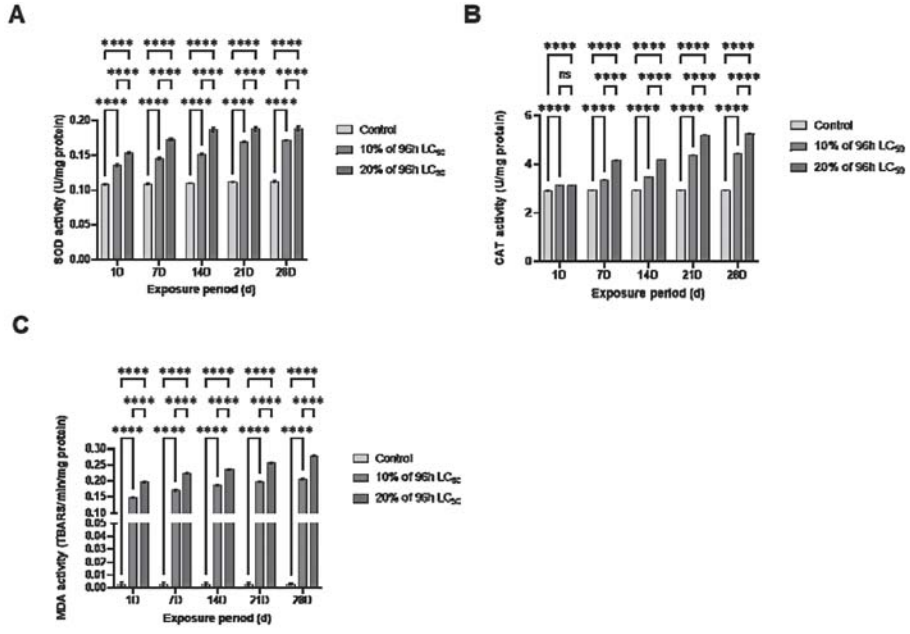


Figure 2. Bar diagrams represent the level of antioxidant enzymes (A) SOD and (B) CAT and oxidative stress biomarker (C) MDA in the hepatopancreas tissue of control and treated groups (10% and 20% of the 96 h LC50) of formic acid exposed *B. bengalensis*. The star (*) on the error bars indicates significant ($p < 0.05$) differences in the values of a particular variable after a one-way ANOVA test. SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase.

defense system, ROS generation in oxidative stress leads to the unsaturated fatty acid degradation in the cell membrane, resulting in lipid peroxidation[27]. Malondialdehyde (MDA) is the end product of lipid peroxidation reaction, which acts as a biomarker for oxidative damage[28]. So, an increased level of MDA has also been observed in formic acid exposed snails, compared to the control group (Fig. 2C).

Integrated Biomarker Response (IBR) and Biomarker Response Index (BRI): The assessment of various oxidative stress enzymes level such as superoxide dismutase (SOD), catalase (CAT) act as the biomarkers for toxicological study[29]. The consideration of a single biomarker may give limited information about the toxicity of environmental pollutants on exposed organisms. So an integrated biomarker response (IBR) is applied to study the organism's response to a toxic substance[30-32]. It is a very useful tool to determine the harmful effects of different pollutants on an organism that is exposed to this environment[31]. It summarizes the changes in all the biomarkers into a single point and helps in evaluating the oxidative stress[32]. An IBR value in the star plot showed that T2-28d is the most affected group (Fig. 3A). IBR values ranged from 0 in control up to 3.54 in T2-28d formic acid exposed snails. According to this index, the rank of the most affected group could be ordered as: T2-28d >T2-21d >T2-14d >T1-28d >T2-7d >T1-21d >T2-1d > T1-14d > T1-7d > T1-1d > control (Fig. 3A).

Biomarkers score of exposed snail groups was used and integrated to analyze the BRI to measure the animal's health condition[30,22]. The BRI value range from 0-1.86, which denotes alteration from normal condition (Fig. 3B). Therefore, it is clear from this analysis, that the health condition of the snails was severely affected due to formic acid exposure.

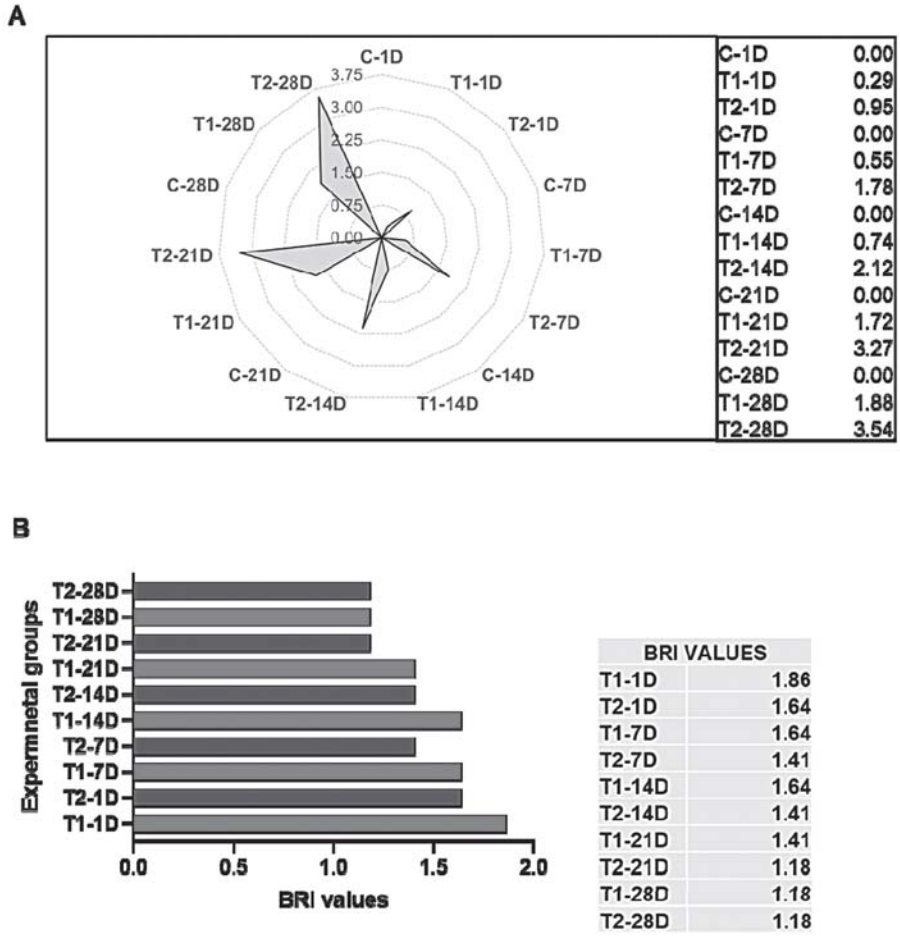


Figure 3. (A) Integrated biomarker response (IBR) of oxidative stress parameters measured in *Bellamiya bengalensis* after chronic exposure to different sublethal doses (10% and 20% of the 96 h LC50) of formic acid. C indicates control (0 mg/l), T1 indicates formic acid concentration at 10% of its 96 h LC50 value; T2 indicates formic acid concentration at 20% of its 96 h LC50 value. (B) Biomarker response index (BRI) of biomarker score measured in *Bellamiya bengalensis* after chronic exposure to different sublethal doses (10% and 20% of the 96 h LC50) of formic acid.

Species Sensitivity Distribution: The species sensitivity distribution (SSD) curve was generated to understand the toxic impact of formic acid on *B. bengalensis* in aquatic habitat as well as to evaluate the proportion of other aquatic species affected due to this toxicity. In the field of toxicological study, SSD is a bell-shaped distribution curve that represents the sensitivity ranges of different species to a particular chemical component[33]. This analysis helps to set the safe limit of the chemical component in a particular environment for the protection and management of that environment[22]. The aquatic species taken for this study, showed a wide range of sensitivity to formic acid, necessitating this complicated interpretation i.e. SSD[22]. Among the aquatic organisms taken for SSD analysis in formic acid exposure according to their 96 h LC50 values, the most sensitive species was

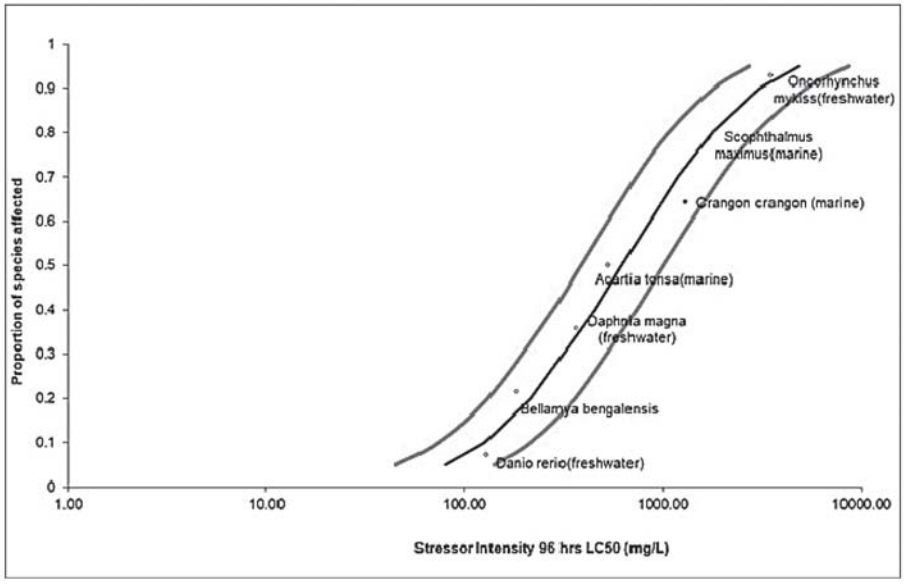
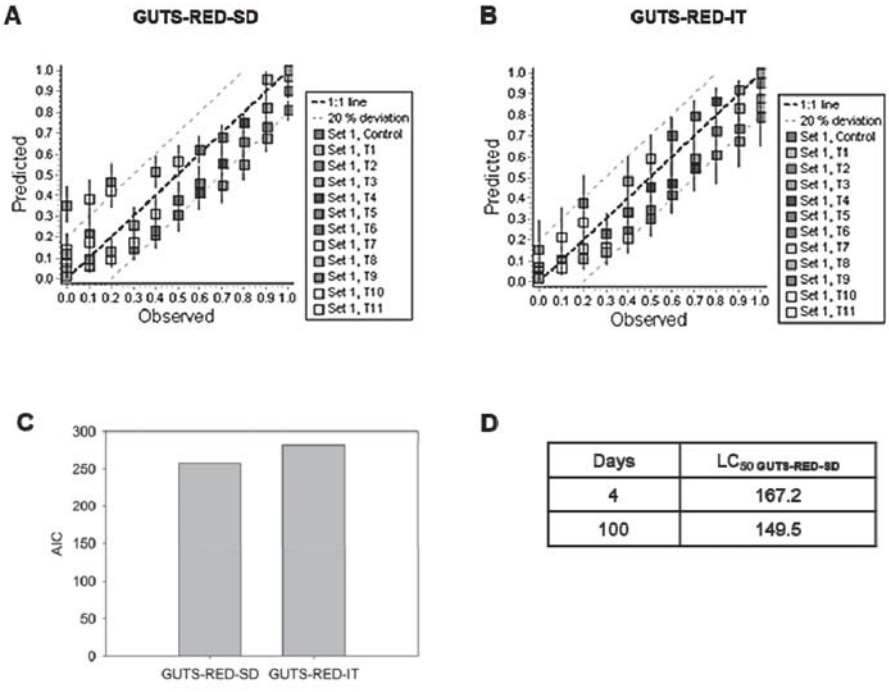


Figure 4. Species sensitivity distribution curve for formic acid. The black line denotes central tendency and the orange lines denote 95% confidence intervals.

Danio rerio, followed by *Bellamya bengalensis*. *Daphnia magna* and *Acartia tonsa* also showed very much sensitivity towards this organic acid (Fig. 7). However, less sensitivity was observed in *Oncorhynchus mykiss*, *Scophthalmus maximus*, and *Crangon crangon*, respectively (Fig. 4).



Days	LC_{50} GUTS-RED-SD
4	167.2
100	149.5

Figure 5. Comparison of the parameters estimated by general unified threshold model (GUTS). Observed vs. Predicted survival plot of formic acid for the calibration of (A) GUTS-RED-SD (B) GUTS-RED-IT model. (C) Comparison of AIC (Akaike Information Criterion) value obtained from GUTS-survival-stochastic death (GUTS-SD) and GUTS–individual tolerance (GUTS-IT) modeling for formic acid. (D) Predicted LC_{50} values by GUTS modeling for 4 days and 100 days.

GUTS Modeling: A toxicokinetic-toxicodynamic (TKTD) study was performed to assess the survival rate of *B. bengalensis* in different exposure concentrations of formic acid. The General unified threshold model of survival (GUTS) was used here, which represents a mathematical framework of survival analysis. Two survival models, i.e. stochastic death (SD) and individual tolerance (IT), were projected to select the best-fitted model for survival analysis[34]. For the GUTS-SD stimulation analysis, the survival rate fits well at 0 mg/l for all concentration formic acid but is overestimated at 230 mg/l, 240 mg/l, 250 mg/l and 260 mg/l whereas the rate of survival was underestimated at 160 mg/l, 170 mg/l, 180 mg/l, 190 mg/l, 200 mg/l and 210 mg/l (Fig. S1A). Furthermore, for GUTS-IT model simulation, the survival rate fits precisely at 0 mg/l for all concentrations and the survival rate was underestimated at 150 mg/l, 160 mg/l, 170 mg/l, 180 mg/l, 190 mg/l, 200 mg/l, 210 mg/l, 220 mg/l (Fig. S1B).

The best-fitted model was selected based on the akaike information criterion (AIC) values (Fig. 5C). A lower value of AIC indicates better fitness. In this study, the GUTS-SD model is better fitted than the GUTS-IT model for formic acid, which explains that the GUTS-SD model can predict the LC50 rate more accurately (Fig. 5C). GUTS-SD model has predicted the 4 days LC50 value as 167.2 (Fig. 5D), which is comparable with the experimental LC50 value. GUTS model also predicted the 100 days LC50 value as 149.5 (Fig. 5D). As, *B. bengalensis* is one of the most sensitive species of formic acid exposure in the aquatic ecosystem, therefore this TKTD study will be very beneficial for understanding the regulatory acceptable

concentration (RAC) of formic acid in an aquatic ecosystem.

Discussion and Conclusion

The present investigation provides a mechano-toxicological understanding of formic acids to a very common aquatic invertebrate *Bellamya bengalensis.* The LC50 values of *B. bengalensis* to this toxicant obtained at 24, 48, 72 and 96 h exposure were 207.32, 199.22, 191.73, 182.69 mg/l respectively (Table 1). Compared to the 96 h LC50 values of other aquatic organisms, *B. bengalensis* appeared as a very sensitive species. Some aquatic organisms showed lower sensitivity to this toxicant. This differential sensitivity among aquatic species to a particular toxicant explains the variable adaptive capacity of an organism to cope with a particular environmental stressor.

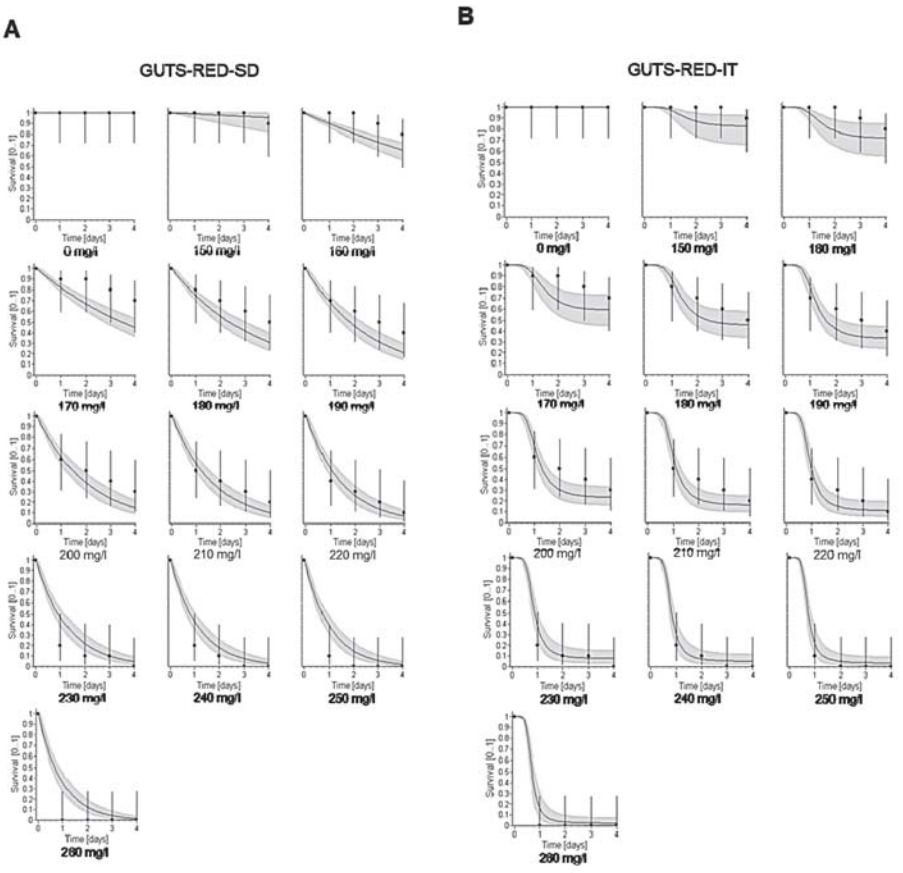


Figure S1. (A) and (B) Comparison of the observed and fitted survival plots for GUTS-RED-SD and GUTS-RED-IT models with different concentrations of formic acid.

In the present study, exposure to different concentrations and periods of formic acid to *B. bengalensis* altered the behavioral pattern like, crawling movement, tentacle movement, touch reflex and mucous secretion explaining the physiological stress due to toxicant exposure. Studies have already shown that locomotion is often impaired in exposure to a toxic environment. Locomotion in an animal is critical for searching for food, reproduction and escape from predators. Therefore, any change in the normal locomotion may affect the organism's fitness, which can influence at population, community and ecosystem levels[35]. In this present study, the reduced crawling movement with increasing concentration and time of formic acid exposure explains the concentration-dependent inhibition in mobility. Snails withdraw their tentacles when touch by obstacles. So, the reduced tentacle movement and touch reflex with increasing formic acid concentration and exposure time suggest degenerative changes in the sensory epithelia[36]. Mucous secretion is one of the physiological mechanisms of snails during exposure to the toxic chemicals to avoid body fluid loss and tissue damage and to prevent any parasitic infection. Mucus also serves as a protective sheath that prevents the direct contact of toxins to the body epithelial layer[37]. So, the increasing mucus secretion in formic acid exposure denotes the severe toxicity and tissue damage in *B. bengalensis.*

Toxic chemicals can alter the physiological homeostasis of an organism by inducing oxidative stress through ROS production. Excessive ROS generation in the tissue is accompanied by to damage proteins, lipids, and DNA structure[38,39]. The cellular antioxidant defense system help in scavenging the free radicals generated due to ROS production[40]. Therefore, checking the level of these antioxidant defense enzymes serve as the biomarker for oxidative stress generation during chemical exposure[41]. SOD and CAT work as the first line of defense enzymes against ROS production. SOD is an enzyme that helps in the breakdown of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2)[31,42]. CAT enzyme functions in the conversion of H_2O_2 into water and O_2 [43,44]. Therefore, both the enzymes SOD and CAT work in a tandem manner and the H_2O_2 produced by the SOD enzyme is removed by CAT. So, these enzymes help in scavenging the free radicals generated during oxidative

stress. In the case of formic acid exposed snails, the SOD level increased compared to the control set, which confirms that organic acid toxicity produces superoxide radicals in the tissue[45]. The increased level of CAT is also because of neutralizing the ROS, generated during organic acid exposure[30]. MDA is an end-product of lipid peroxidation reaction in the plasma membrane due to ROS production[28]. In this present investigation, the MDA level in treated snails increased in a concentration and time-dependent manner. Increased MDA level suggests more ROS production that leads to protein and lipid degradation in the snail tissue. Therefore, a significant increase in the level of SOD and CAT and lipid peroxidation indicator MDA level upon formic acid exposure to the snails supports the explanation of oxidative burst.

The integrated biomarker response (IBR) was applied to calculate the overall oxidative stress generated due to formic acid exposure. The greater IBR value compared to the control represents the stressful environmental condition in a toxic environment whereas a lower value indicates a favourable condition[1,46]. IBR analysis showed that 28 days of exposure to formic acid result in the highest oxidative stress to the animals.

In the present study, IBR results represent that 28 days of exposure to 20% of LC50 value in formic acid is the most affected group. So, this IBR result explains that a higher concentration of formic acid on long-term exposure causes more oxidative stress to the snails. In addition, during this current study, a biomarker-based index (BRI) is also used to monitor the health status of the animals during formic acid exposure. This result also depicted an adverse health condition of the animals due to formic acid toxicity.

Ethical Approval

This article contains no animal studies by any of the authors requiring endorsement from the ethical committee. In fact, according to the current regulatory studies, no ethical authorization is required for invertebrates like *Bellamiya bengalensis*.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the authors upon reasonable request.

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