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Neuro-genotoxicity assessment of sublethal exposure of carbosulfan to freshwater Fish, *Cyprinus carpio* (L.)

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Key words: Carbosulfan, *Cyprinus carpio*, Neurotoxic, Micronucleus assay, Comet assay

Abstract

Carbosulfan, a carbamate pesticide extensively employed in rural communities, enters the aquatic environment by the proximity of agricultural lands to water bodies or through direct application in such environments. The study's goal was to investigate the neurotoxic effects of carbosulfan using ACh and AChE levels in brain tissue, and genotoxic effects using Micronucleus (MN) assay in blood cells and Comet assay in gill cells of *Cyprinus carpio*. The fish was exposed to $1/5^{th}$ & $1/10^{th}$ sublethal concentrations of 96 h LC_{50} for 7, 14, and 21 days. There were significant ($P<0.05$) alterations in ACh and AChE content and carbosulfan was induced to show MN formation and DNA damage in concentration and time-dependent manner. The reduced ionic composition in *C.carpio* brain tissue may explain the inhibition of AChE and the rise of ACh concentration. The significant increase in MN and DNA damage observed in carbosulfan-exposed fishes in the current study indicates the mutagenic/genotoxic potential of carbosulfan in the freshwater fish *C.carpio*, as well as the potential value of the Common carp for assessing pesticide pollution of freshwater bodies. Changes in these characteristics may provide an early warning signal for determining pesticide toxicity and its impact on aquatic species. As a result, it is necessary to monitor the aquatic system and forecast the hazardous effect of carbosulfan on fish; precautions should be taken while using even low concentrations of carbosulfan, and prohibiting or restricting carbosulfan usage is preferable.

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Introduction

Carbosulfan is an insecticide used for the control of insects, mites, and nematodes on potatoes, sugar beets, rice, maize, and citrus. It is exceedingly toxic to fish, and its toxicity is mediated in the nervous system through acetylcholinesterase inhibition (Yi *et al.*, 2006). Carbosulfan, which is commonly used in rural communities, penetrates the aquatic environment by the proximity of agricultural lands to water bodies or directly through reckless application in such environments and affects aquatic species, and its concentrations in surface and groundwater are predicted to be between 29µg/L and 0.64µg/L (Leppert *et al.*, 1983; Sao *et al.*, 2008). It is converted to carbofuran in animals by hydroxylation or oxidation processes in water (Giri *et al.*, 2003) and has been restricted or banned in some countries, primarily due to the formation of highly toxic metabolites. Pollution from pesticides in water kills fish and other aquatic creatures (Svensson *et al.*, 1994). Fish are particularly sensitive to environmental changes. Thus, fish health may reflect and be an excellent predictor of the overall health of an aquatic environment (Burkepile *et al.*, 2000). Even though carbosulfan is not stable in water and does not remain in the environment, fish bioaccumulate to some amount due to their slower metabolism. Its high water solubility, widespread use in the environment, and exposure to non-target creatures may all offer long-term risks to aquatic organisms (IPCS, 1986).

The present study investigates the neurotoxic effects of carbosulfan using ACh and AChE levels in brain tissue, and genotoxic effects using Micronucleus (MN) assay in blood cells and Comet assay in gill cells of *Cyprinus carpio* exposed *in vivo*. Because of the reduction of its activity, acetylcholinesterase (AChE; enzyme classification 3.1.1.7) is widely recognized as a particular biomarker of carbamate pesticides (Fairbrother and Bennett, 1988). By hydrolyzing the ubiquitous neurotransmitter acetylcholine, this enzyme modulates neuronal transmission in the synaptic cleft. AChE deficiency causes central and peripheral nervous system problems as well as mortality (Quinn, 1987).

Many studies have shown that the micronucleus (MN) test and the comet assay (CA) are the two most sensitive, rapid, and widely used methods for detecting the genotoxicity of chemicals and xenobiotics in the field and laboratory (Ateeq *et al.*, 2002; Pandey *et al.*, 2006). Despite the fact that carbosulfan has been shown to induce micronuclei, sister chromatid exchange, and chromosomal aberrations in human peripheral blood lymphocytes and rat bone marrow cells (Sterhrer-Schmid and Wolf, 1995; Topaktas *et al.*, 1996; Rencüzogullari and Topaktas, 2000; Giri *et al.*, 2003), research on the genotoxic properties of carbosulfan in aquatic organisms is few, particularly data on its effects on fish. The experimental fish, *Cyprinus carpio* (L.) is a cool to temperate water fish species that, due to its economic importance and status as a major element of many food chains around the world, is an ideal model indicator for toxicological investigations.

Materials & methods

Experimental fish specimen and chemical

Cyprinus carpio (L.) (Family: Cyprinidae, Order: Cypriniformes) was obtained from Fishery Department, B.R. Project, Shimoga, Karnataka state. The specimens weighed 3.5 to 4.5g and measured 5 to 6cm long. To minimize cutaneous infections, fish specimens were washed twice for 2 minutes in 0.05% potassium permanganate ($KMnO_4$). The specimens were subsequently acclimatized in semi-static systems under laboratory conditions for two weeks. During the acclimatization period, the fish were fed commercial fish pellets twice a day. Feces and other waste items were siphoned off daily to lower the ammonia concentration of the water. Commercial formulations of carbosulfan (25% EC) with the trade name 'Marshal', manufactured by FMC India Private Limited, were purchased from the local market for the current study. Physiochemical parameters of water were measured during the test (APHA, 2005).

In vivo exposure experiment

Acute toxicity was carried out in semi-static conditions by OECD guideline No. 203 (OECD, 1992).

The LC_{50} and 95% confidence limits of carbosulfan for *Cyprinus carpio* were calculated using a basic program from the Probit analysis described by Finney (1971), and the 96 h LC_{50} value of carbosulfan to *C.carpio* was found to be 3.41mg/L. $1/5^{th}$ (0.68mg/L) - SL-1 and $1/10^{th}$ (0.34mg/L) - SL-2 of LC_{50} were chosen as the nominal sublethal concentrations and were employed in the current study to examine the neurotoxic effects of carbosulfan using ACh and AChE assay in brain tissue, and genotoxic effects using Micronucleus (MN) assay in blood cells and Comet assay in gill cells of *C.carpio*. The fish specimens were exposed to these two test concentrations in a semi-static system with the change of test water on every alternate day to maintain the concentration. The exposure was continued for up to 21 days and tissue sampling was done during exposure periods of 7, 14, and 21 days.

Neurotoxicity experiments

Estimation of acetylcholine (ACh) content

The tissue ACh content was calculated using Hestrin's technique, as reported by Augustinson (1957). After separating and weighing the brain tissue, it was teased and transferred to tubes that had already been placed in a boiling water bath for 10 min to inactivate the enzyme acetylcholinesterase and release bound ACh. After cooling the tubes, the contents were homogenized in 2.0ml of distilled water. A total of 2.0ml of alkaline hydroxylamine hydrochloride and 1.0ml of dilute HCl with H_2O at a ratio of 1:1 were added. The contents were centrifuged, and the supernatant was treated with 1.0ml of ferric chloride. The optical density of the sample was measured in a spectrophotometer at 540nm in comparison to a blank.

Estimation of acetylcholinesterase (AChE) activity

Metcalf's (1951) procedure was used to calculate acetylcholinesterase activity. In cold 0.25M sucrose solution, 3% of the homogenate of brain tissue was produced and homogenated. The enzyme test was performed using supernatant. A total of 3.0ml of reaction mixture included 12 µm of acetylcholine chloride, 100 µm of sodium phosphate buffer (pH 7.4), and 1.0ml of homogenate. After 30 minutes of incubation at 37°C, the reaction was halted by adding 2.0ml of alkaline hydroxylamine hydrochloride solution, followed by 1.0ml of HCl (1:1 HCl: H_2O). The mixture was properly blended and filtered. 1.0ml of 0.37 M ferric chloride solution was added to the clear filtrate, and the color was measured at 540 nm in a spectrophotometer with a blank. Protein content in each homogenate was estimated (Lowry *et al.*, 1951) to determine the specific activity of the enzyme.

Genotoxicity experiments

Micronucleus (MN) Assay

The MN test was carried out using the methodology of Grisolia and Cordeiro (2000), with slight changes. Blood samples were spread on a clean glass slide, air dried, and then fixed for 15 min in absolute methanol. For 10 min, each slide was stained with 5% Giemsa solution. The stained cells were examined and scored under a microscope.

To determine the frequency of cells with one, two, or more two micronuclei, at least 1000 binucleated cells per duplicate cell culture are scored. The major criteria for scoring the micronucleus (MN) were based on those of Al-Sabti and Matcalfe (1995), which included the lack of connections with the main nucleus, identical color, and size of $1/10^{th}$ to $1/30^{th}$ of the main nucleus. According to Ayllon and Garcia-Vazquez (2000), the nuclear abnormalities detected were divided into categories viz., a) micronuclei, b) binucleated nucleus, c) lobed nucleus, and d) other nuclear abnormalities. The MN frequencies (and all other parameters) were calculated as

$$\% \text{ MN} = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

Comet Assay

With minor changes (Klaude *et al.*, 1996), the alkaline single-cell gel electrophoresis (SCGE)/comet test was done as a three-layer technique (Singh *et al.*, 1988). The gill tissue was homogenized in an ice-cold homogenization buffer (1X Hanks' balanced salt solution (HBSS), 20mm EDTA, 10% dimethyl sulfoxide (DMSO), pH 7.0-7.5), then centrifuged at 3000rpm for 5 minutes at 4°C.

After that, the cell pellet was suspended in cooled phosphate-buffered saline (PBS). The viability of gill cells was determined using the trypan blue exclusion test technique (Anderson, *et al.*, 1994), and tissue samples with cell viability of more than 84% were processed for the comet assay. In summary, up to 15µL of cell suspension (approximately 20,000 cells) was combined with 85µL of 0.5% low melting point agarose (LMPA) and stacked on one end of a frosted glass slide before being covered with 200µL of 1% normal agarose. The third coat of 100µL LMPA was applied. After the gel had solidified, the slides were submerged in a lysing solution (2.5 M NaCl, 100mm Na_2-EDTA, 10mm Tris, pH 10, with 10% DMSO and 1% Triton X- 100 added fresh) overnight at 4°C. Following lysis, the slides were placed side by side in a horizontal electrophoresis unit containing fresh cold alkaline electrophoresis buffer (300mm NaOH, 1mm Na_2-EDTA, and 0.2% DMSO, pH 13.5) and left in the solution for 20 minutes at 4°C to allow DNA unwinding and conversion of alkali-labile sites to single-strand breaks. The same alkaline electrophoresis buffer was used for alkaline electrophoresis for 20 minutes at 15 V (0.8 V/cm) and 300 mA at 4°C. To remove the excess alkali, the slides were washed three times for 5 minutes in a neutralization buffer (0.4 M Tris, pH 7.5). 75µL ethidium bromide (20µg/ml) was used to stain the slides. The cells were randomly scored and analyzed with an image analysis system (Komet - 5.5 software) attached to a fluorescent microscope (Nikon E600) equipped with appropriate filters. As determined by the software, the parameter chosen for quantification of DNA damage was% Tail DNA (% tail DNA = 100 -% head DNA).

Statistical Analysis

To examine ACh & AChE levels, and the mean differences in MN frequencies (and all other parameters),% tail DNA between concentrations within the tissue, and between durations within concentrations were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple range tests, and statistical significance was assessed at 5% ($P < 0.05$) levels using GraphPad version (8.0) software.

Results

There were no fish deaths during the acclimatization period before carbosulfan exposure, no fish died throughout the *in vivo* exposure experiment and all of the given feed was swiftly devoured by fish in all treatments. Carbosulfan had a considerable impact on the fish's growth rate. Fish exposed to carbosulfan gained 3% of their body weight at the end of the experiment, whereas those in the control group gained 10% of their body weight.

Physico-chemical parameters of the test water

The physicochemical properties of the water used in the experiment were as follows: Temperature 24±1°C, pH 7.4±0.3 at 25°C, Dissolved Oxygen 6.9±0.54mg/L, Carbon-dioxide 5.9±0.6mg/L, Total Hardness 22.4±2.4mg as $CaCO_3$/L, Total ammonia nitrogen 0.02mg/L, Phosphate 0.35±0.004µg/L, Salinity 0.01ppm, Specific Gravity 1.001 and Conductivity less than 10µS/cm.

Neurotoxicity experiments

ACh

Since $P<0.05$ there is a significant increase in the ACh level of brain tissue of *C.carpio* with the different days of exposure in both the exposure concentrations. Increased concentrations of carbosulfan from SL-2 to SL-1 show significant changes in ACh concentrations (Fig.1).

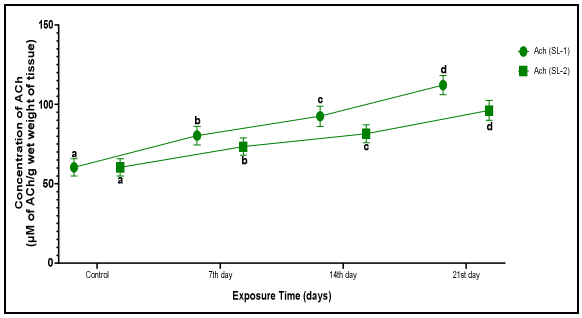

Fig. 1. Changes in the ACh content in brain tissue of *Cyprinus carpio* exposed to sublethal concentrations (SL-1 & SL-2) of carbosulfan for 7, 14, and 21 days. Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of ACh/g wet weight of tissue. Different alphabets within each concentration between exposure times denote significance at a 5% level ($P<0.05$) and the same alphabets denote non-significance.

AChE

Since P<0.05 there is a significant decrease in the AChE level of brain tissue of *C. carpio* with the different days of exposure in both the exposure concentrations. Increased concentrations of carbosulfan from SL-2 to SL-1 show significant changes in AChE concentrations (Fig. 2).

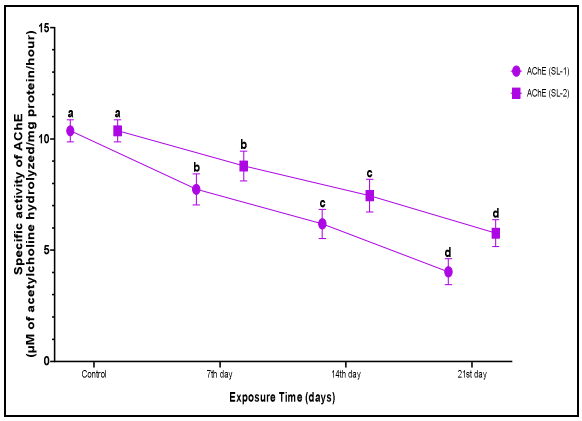

Fig. 2. Changes in the AChE content in brain tissue of *Cyprinus carpio* exposed to sublethal concentrations (SL-1 & SL-2) of carbosulfan for 7, 14, and 21 days. Each datum represents the Mean ± SD, of six individuals (n=6). Values are expressed as µM of acetylcholine hydrolyzed/mg protein/hour. Different alphabets within each concentration between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance.

Genotoxicity experiments

MN assay

In comparison to the control, the carbosulfan concentrations resulted in a significant increase in MN (t-test). Carbosulfan also caused a dose-dependent increase in MN, with the greatest frequency of MN recorded at the SL-1 concentration and increased concentrations of carbosulfan from SL-2 to SL-1 showing significant changes in MN formation (Fig. 3). At the two carbosulfan concentrations, MN increased in a time-dependent manner. These results are the same for the binucleated nucleus, lobed nucleus, and other nuclear abnormalities that were detected during sublethal exposure.

Comet Assay

During electrophoresis, DNA migration from gill cells resulted in the production of comets, and the DNA damage was quantified as tail length. The length of a tail is related to the amount of DNA damage. The comet test demonstrated that DNA damage in fish exposed to the carbosulfan is time and concentration-dependent in the current investigation (Fig. 4 & 5). Maximum tail length was assessed for a certain time of exposure in the case of SL-1, followed by SL-2, but for both concentrations, maximum tail length was found on the 21st day (Fig. 4 & 5) and increased concentrations of carbosulfan from SL-2 to SL-1 shows significant changes in DNA damage.

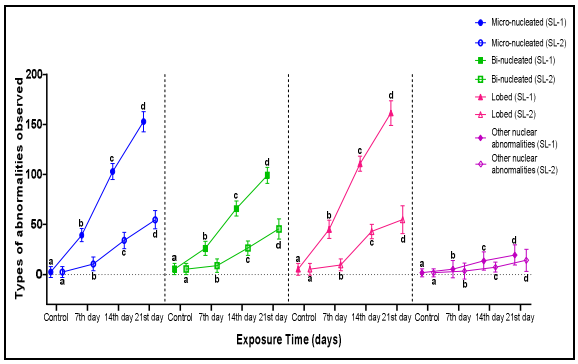

Fig. 3. MN and other abnormalities induced by sublethal concentrations (SL-1 & SL-2) of carbosulfan in *C.carpio* blood cells at 7, 14, and 21 days. Each datum represents the Mean ± SD, of six individuals (n=6). Different alphabets within each concentration between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance.

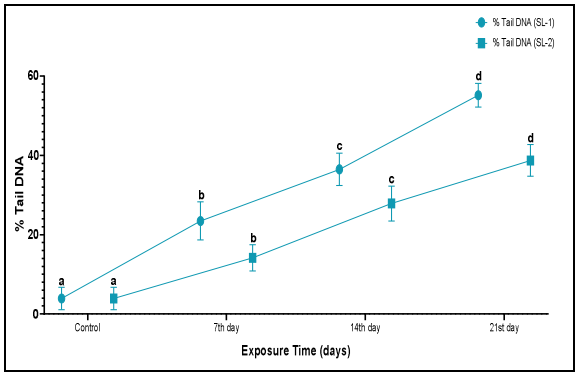

Fig. 4. DNA damage (% Tail DNA) induced by sublethal concentrations (SL-1 & SL-2) of carbosulfan in *C.carpio* gill cells at 7, 14, and 21 days. Each datum represents the Mean ± SD, of six individuals (n=6). Different alphabets within each concentration between exposure times denote significance at a 5% level (P<0.05) and the same alphabets denote non-significance.

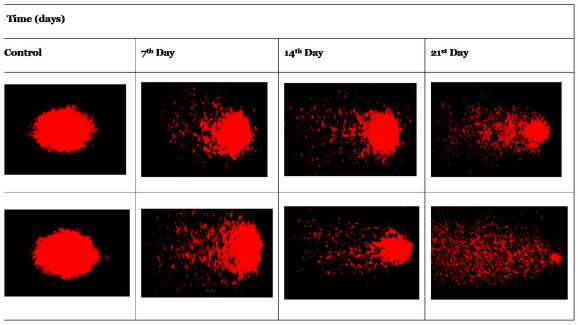

Fig. 5. Comets formed in gill cells of *C.carpio* exposed to carbosulfan.

Discussion

Toxicity testing is an important component in assessing the impact of pesticides on aquatic environments since it reveals hazardous qualities of chemicals in organisms by modifying their behavior and survival rate (Kaushal and Misha, 2013). It provides baseline data on the effects of pesticides on aquatic organisms, especially fish, as well as the ecosystem as a whole. These studies are critical in raising awareness about the potentially negative impacts of pesticides on the environment (Adedeji *et al*., 2008).

Neurotoxicity experiments

Acetylcholinesterase is an enzyme that regulates the amount of neurotransmitter material present at neuron junctions (O'Brien, 1967), as well as the ionic content (Van der Kloot, 1956). Neurotransmitters are required to keep nerve impulses flowing from one nerve cell to the next across the synaptic gap. AChE deactivates ACh nearly soon after the impulse is conveyed by breaking it down. If AChE is blocked, ACh, accumulates and nerve impulses cannot be halted, resulting in, paralysis, and also causes behavioral abnormalities and widespread disruption in normal physiology, ultimately leading to the organism's death. In the current study, the reduced ionic composition in *C.carpio* brain tissue may explain the inhibition of AChE and the rise of ACh concentration. (Fig. 1 & 2) indicating a reduction in cholinergic transmission and, as a result, a buildup of ACh in the tissue and implies stronger inhibition of central nervous system integratory activity (Reddy *et al*., 1992). Damage to the central nervous system may have resulted in uncontrolled hormone release, and an animal's toll may be conceivable due to the degradation of numerous biochemical and physiological activities (Corbett, 1974). The results agree with the findings of (Capkin *et al*., 2014) which show the inhibitory effect of carbosulfan on AChE in rainbow trout fish and a comparable corroborative rise in ACh content as a result of a drop in tissue AChE levels due to deltamethrin exposure on *C.carpio* was documented in (Adinarayan and Kishore, 2018).

Genotoxicity experiments

Fish are an effective model organism for evaluating contaminants' harmful and mutagenic potential in water samples (Belpaeme, 1996). The significant increase in MN and DNA damage observed in carbosulfan-exposed fishes in the current study indicates the mutagenic/genotoxic potential of carbosulfan in the freshwater fish *C.carpio*, as well as the potential value of the Common carp for assessing pesticide pollution of freshwater bodies. Although the Comet test reveals DNA strand-breaking activity, most DNA strand breaks may be easily repaired. The MN test is associated with the production of DNA damage and genomic instability, and it identifies genotoxic insults. A combination of the MN test and Comet assay demonstrates the possibility of single and double-strand breakage caused by carbosulfan in *C. carpio* blood and gill cells. As a result, it is advised that these two criteria be used combined to confirm the test agent's overall mutagenic/genotoxic impact.

In the current study, blood and gill cells from carbosulfan-exposed fish showed a significant increase in MN and DNA damage respectively (Fig. 3, 4 & 5). Few previous studies (Altinok *et al*., 2012; Nwani *et al*., 2010) have verified the use of carbosulfan as a genotoxicant and positive mutagen.

Micronuclei are the result of chromosomal abnormalities caused by preceding mitotic divisions in erythrocytes (Matter and Grauwiler, 1974). The formation of a micronucleus as a result of a cell cycle error suggests that it may play a role in tumor formation, leading to a cancerous state (Jayashree, 1994). In the present study, the increased MN in blood cells may be caused by the interaction of

carbosulfan with chromosomes by targeting nucleophilic sites on DNA and carbosulfan-induced DNA double-strand breaks, which result in symmetrical/ asymmetrical chromatid and chromosome exchanges or fragments that fail to be included in the daughter nuclei at the end of telophase due to a lack of spindle attachment during the anaphase segregation process and the dose-dependent rise in the incidence of MN may be owing to larger doses of carbosulfan inducing more double-strand DNA breaks and their lower chance of being properly repaired.

Carbosulfan-induced DNA damage in *C. carpio* gill cells may be attributed to the formation of reactive oxygen species (ROS). ROS generated in excess is harmful to both the cell and DNA (Cadet *et al*., 2003), producing breaks in the DNA strands (Reinecke and Reinecke, 2004) and may cause apoptosis (Nwani *et al*., 2011). Increased ROS generation as a result of xenobiotic-induced toxicity causes lipid peroxidation of bio-membranes, affecting their permeability and integrity and possibly causing DNA damage (Hulbert *et al*., 2007). And the intermediates generated during carbosulfan catabolism may potentially interact with intact DNA molecules, causing structural damage. It has also been proposed that labile methyl groups in pesticides would be an excellent substrate for electrophilic assault, resulting in the methylation of the DNA to behave as DNA lesions (Wang *et al*., 2008). Furthermore, the DNA damage found in the present research might have been caused by single-strand breaks, double-strand breaks, DNA adduct formation, and DNA-DNA and DNA-protein cross-links (Mitchelmore and Chipman, 1998).

Conclusion

There were significant ($P<0.05$) alterations in ACh and AChE content and carbosulfan was induced to show MN formation and DNA damage in concentration and time-dependent manner. The reduced ionic composition in *C.carpio* brain tissue may explain the inhibition of AChE and the rise of ACh concentration. A combination of the MN test and Comet assay demonstrates the possibility of single and double-strand breakage caused by carbosulfan in *C.carpio* blood and gill cells, as a result, it is advised that these two criteria be used combined to confirm the test agents' overall mutagenic/genotoxic impact. Changes in these characteristics may provide an early warning signal for determining pesticide toxicity and its impact on aquatic species. As a result, it is necessary to monitor the aquatic system and forecast the hazardous effect of carbosulfan on fish; precautions should be taken while using even low concentrations of carbosulfan, and prohibiting or restricting carbosulfan usage is preferable.

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Author contributions

Krishnamurthy Yogesh: Data Curation; Investigation; Methodology; Writing - original draft.
Mididoddi Venkateshwarlu: Supervision; Writing - review & editing.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

Disclosure of interest

The authors report there are no conflicts of interest to declare.

List of abbreviations

EC: Emulsifide Concentration; FMC; Food Machinery & Chemical Corporation; APHA: American Public Health Association; OECD: Organization for Economic Cooperation and Development; LC_{50}: Lethal concentration-50; SL-1: Sublethal concentration 1; SL-2: Sublethal concentration 2; ACh: Acetylcholine; AChE: Acetylcholinesterase; MN: Micronucleus.

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