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Ecotoxicological assessment of Benzalkonium Chloride-induced behavioral and histological changes in *Cyprinus carpio* (L.)

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Abstract
The extensive use of household and industrial cleaning products has led to increased environmental pressure on aquatic ecosystems, with substances like benzalkonium chloride posing potential hazards to aquatic life. This study examines the xenobiotic effects of benzalkonium chloride on the gill and muscle tissues of the freshwater fish *Cyprinus carpio* (common carp). Fingerlings (average weight: 4.3 ± 1.4 g; length: 5.1 ± 1.7 cm) obtained from a fish farm in Tumkur, Karnataka, were used to evaluate acute toxicity through static bioassay experiments, including determination of the 24-hour LC_{50} value. The fish were exposed to varying concentrations of benzalkonium chloride, and behavioral responses along with mortality were monitored at 2-hour intervals. Biochemical assessments indicated significant changes in glucose, glycogen, and protein levels in both gill and muscle tissues. Glycogen and protein contents showed a notable decline, while glucose levels increased in comparison to the control group, suggesting elevated metabolic stress and energy requirements. The depletion of glycogen may reflect its utilization to meet heightened energy demands under toxic conditions, whereas reduced protein levels indicate increased protein breakdown. Histological observations revealed considerable damage to gill structures, including epithelial desquamation, necrosis (telangiectasia) of secondary lamellae, lamellar shrinkage, and hypertrophy and hyperplasia at the lamellar base. Muscle tissue analysis also demonstrated significant alterations such as nuclear proliferation, irregular fiber size, degeneration and atrophy of muscle bundles, and separation with thickening of fibers. Overall, the study indicates that benzalkonium chloride causes pronounced biochemical and histological changes in *Cyprinus carpio*, particularly in early life stages, emphasizing its ecological significance and the need for regulated use of such contaminants in aquatic environments.

Keywords: *Cyprinus carpio*, Benzalkonium chloride, desquamation, hyperglycemia, necrosis, histopathology, biochemical alterations

Introduction
Background
In recent decades, the rapid increase in the use of household and industrial chemicals has significantly contributed to environmental contamination, particularly in aquatic ecosystems (Fent *et al.*, 2006) [4]. Among these, quaternary ammonium compounds such as benzalkonium chloride (BAC) are widely used as disinfectants and surfactants in cleaning agents, pharmaceuticals, and personal care products (Li *et al.*, 2020). Due to their extensive application, these compounds frequently enter aquatic systems through domestic wastewater, industrial discharge, and improper disposal practices (APHA, 2017).
Benzalkonium chloride exhibits strong surface-active properties that enable it to disrupt microbial cell membranes, making it highly effective as a disinfectant. However, these same properties can adversely affect non-target aquatic organisms. Recent studies have shown that BAC is increasingly detected in aquatic environments and is considered an emerging contaminant of concern due to its persistence and ecological risks (Wang *et al.*, 2025) [15]. Once released into water bodies, BAC can alter physicochemical properties and disturb ecological balance, ultimately leading to toxic effects in aquatic fauna, including fish (Fent *et al.*, 2006) [4].

Importance of Studying Fish
Fish are widely recognized as effective bioindicators of aquatic pollution due to their sensitivity to environmental stressors and ecological relevance (Van der Oost *et al.*, 2003) [13]. The freshwater fish *Cyprinus carpio* (common carp) is extensively used in toxicological studies because of its adaptability, commercial importance, and well-documented physiology (Rand, 1995) [9].
Among fish organs, the gills and muscles are particularly susceptible to toxicants. Gills are in constant contact with the surrounding water and serve as the primary site for respiration, ion regulation, and excretion, making them highly vulnerable to pollutants (Evans *et al.*, 2005) [3]. Muscle tissue, essential for locomotion and energy storage, reflects metabolic disturbances caused by toxic exposure (Sharma *et al.*, 2012) [10].

Benzalkonium Chloride and Its Environmental Impact
Benzalkonium chloride is a cationic surfactant composed of alkyl benzyl dimethyl ammonium compounds. Despite its effectiveness as an antimicrobial agent, its persistence and bioaccumulation potential raise serious environmental concerns (Li *et al.*, 2020) [5]. Recent research highlights that BAC can induce oxidative stress, disrupt metabolic pathways, and impair physiological functions in aquatic organisms (Zeng *et al.*, 2024) [17].

Additionally, exposure to environmentally relevant concentrations of BAC has been shown to influence gene expression and induce resistance-related responses in fish, indicating its broader ecological impact (Yin *et al.*, 2025) [16]. BAC exposure can also lead to biochemical disturbances and histopathological damage in aquatic species, affecting survival and growth (Sousa *et al.*, 2023) [11].

Objectives of the Study

The present study aims to evaluate the toxicological effects of benzalkonium chloride on the freshwater fish *Cyprinus carpio*, with emphasis on biochemical and histological responses. The specific objectives are:

1. **Determination of LC_{50} Values:** To estimate the median lethal concentration (LC_{50}) of benzalkonium chloride using standard bioassay methods (OECD, 2019).
2. **Assessment of Biochemical Alterations:** To evaluate changes in glucose, glycogen, and protein levels in gill and muscle tissues under toxic stress.
3. **Histopathological Evaluation:** To investigate structural alterations in gill and muscle tissues.
4. **Behavioral Analysis:** To observe changes in swimming behavior, feeding activity, and stress responses.
5. **Oxidative Stress Assessment:** To examine oxidative stress markers and antioxidant defense mechanisms in response to BAC exposure.

Significance of the Study

Understanding the impact of benzalkonium chloride on freshwater fish is essential for evaluating the ecological risks associated with commonly used disinfectants. Recent assessments indicate that BAC poses a measurable ecological risk in aquatic environments, even at low concentrations, emphasizing the need for regulatory monitoring (Wang *et al.*, 2025) [14].

This study contributes to the field of aquatic toxicology by providing insights into biochemical and histological alterations in *Cyprinus carpio*. The findings highlight the necessity for improved wastewater treatment technologies and stricter environmental regulations to mitigate the harmful effects of such contaminants (Fent *et al.*, 2006) [4].

Materials and Methods

Selection of Test Organisms

Fingerlings of *Cyprinus carpio* (average weight: 4.3 ± 1.4 g; length: 5.1 ± 1.7 cm) were procured from a fish farm located in Tumkur, Karnataka. The fish were carefully transported to the laboratory in aerated polyethylene bags to minimize handling stress and physical injury. Upon arrival, the specimens were transferred to large plastic tanks containing dechlorinated water and allowed to acclimatize to laboratory conditions for a period of 10–14 days. During acclimation, fish were maintained under controlled temperature and photoperiod conditions and fed a standard diet consisting of groundnut oil cake and rice bran (APHA, 2017[1]; OECD, 2019).

Bioassay Protocol

Acute toxicity tests were performed using a static bioassay method to evaluate the toxic effects of benzalkonium chloride. Dechlorinated tap water was used for both control and experimental setups. Groups of ten healthy and acclimatized fish were exposed to different concentrations of benzalkonium chloride under laboratory conditions. Feeding was discontinued 24 hours prior to and throughout the experimental period to avoid interference with metabolic activity. The experimental design followed standard guidelines for fish toxicity testing (OECD, 2019) [8]. Each treatment was conducted in duplicate to ensure reproducibility and reliability of the results (Wang *et al.*, 2025) [14].

Determination of 24-Hour Lc_{50}

Commercially available benzalkonium chloride was used as the test toxicant. Various concentrations (0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 ml/L) were prepared in separate containers, each containing 10 liters of water. Ten fish were introduced into each concentration, while a control group without toxicant was maintained under identical conditions. Continuous aeration was provided throughout the experiment, and no feeding was carried out during the exposure period. Mortality was recorded after 24 hours, and dead fish were promptly removed. The median lethal concentration (LC_{50}) was calculated using standard tabular methods (OECD, 2019). Recent studies emphasize that BAC exhibits acute toxicity even at low concentrations, highlighting the importance of LC_{50} determination for ecological risk assessment (Zeng *et al.*, 2024) [17].

Behavioral Observations

Behavioral responses of *Cyprinus carpio* were carefully monitored immediately after exposure and at regular intervals during the experiment. Fish in the control group displayed normal swimming behavior and feeding activity. At lower concentrations of benzalkonium chloride, fish exhibited minimal changes; however, with increasing concentrations, significant behavioral alterations were observed. These included erratic swimming, loss of equilibrium, hyperactivity followed by lethargy, and increased opercular movements. At higher concentrations, fish showed severe stress responses such as lateral resting, reduced responsiveness, and rapid opercular movement with open mouth, indicating respiratory distress. Such behavioral abnormalities are considered early indicators of toxic stress in fish (Sousa *et al.*, 2023) [11].

Biochemical Analysis

At the end of the exposure period, fish were sacrificed, and tissues such as gills and muscles were dissected for biochemical analysis. Glucose levels were estimated using the Anthrone method, glycogen content was determined following Kemp's method, and protein levels were measured using the Lowry method. These biochemical parameters are widely used indicators of metabolic stress and energy utilization in fish exposed to toxicants (Sharma *et al.*, 2012) [10]. Alterations in these parameters reflect the physiological adjustments made by fish to cope with chemical stress, including increased energy demand and protein catabolism (Zeng *et al.*, 2024) [17].

Histological Analysis

For histopathological examination, gill and muscle tissues were excised immediately after sacrifice to prevent autolysis. The tissues were fixed in Bouin's solution, followed by dehydration through a graded ethanol series, clearing in xylene, and embedding in paraffin wax at 56–58°C. Thin sections (4–6 µm) were prepared using a microtome and stained with hematoxylin and eosin. The stained sections were examined under a light microscope to identify structural alterations in tissues. Histological studies provide crucial insights into tissue-level damage caused by toxicants and are widely used in ecotoxicological assessments (Van der Oost *et al.,* 2003; Sousa *et al.,* 2023) [11, 13].

Results

Toxicity Studies

The median lethal concentration (LC_{50}) represents the concentration of a toxicant at which 50% of the test organisms survive within a specified exposure period. In the present study, LC_{50} values were determined by plotting the concentration of benzalkonium chloride on the X-axis against the percentage survival of *Cyprinus carpio* on the Y-axis. A linear relationship was obtained, and the LC_{50} value was identified at the point corresponding to 50% survival.

The 24-hour LC_{50} value of benzalkonium chloride for *Cyprinus carpio* fingerlings was found to be 0.06 ml/L using the tabular method. No mortality was observed in the control group throughout the experimental period, confirming that the test conditions were suitable and free from external stress factors.

At higher concentrations, particularly at 0.06 ml/L, complete mortality (100%) was recorded within 24 hours. Mortality increased progressively with increasing concentrations of benzalkonium chloride, indicating a clear dose-dependent toxic effect. These findings suggest that benzalkonium chloride exhibits significant acute toxicity to *Cyprinus carpio*, even at relatively low concentrations.

Table 1: LC50 value of benzalkonium chloride exposure in Cyprinus carpio

Toxicant	Fish Species	Exposure Period	Method	LC_{50}
Benzalkonium chloride	*Cyprinus carpio*	24 hours	Tabular	0.06 ml/L

Behavioral Studies

Behavioral responses of *Cyprinus carpio* exposed to varying concentrations of benzalkonium chloride were systematically observed during the experimental period. Fish in the control group exhibited normal swimming behavior, equilibrium, and respiratory activity throughout the study.

At the lowest concentration **(**0.01 ml/L**),** no significant behavioral deviations were observed, and fish maintained normal activity. However, as the concentration of benzalkonium chloride increased, distinct behavioral alterations became evident. At moderate concentrations (0.02–0.04 ml/L), fish exhibited erratic swimming patterns, occasional surfacing, and mild respiratory distress characterized by increased opercular movements.

At higher concentrations (0.05–0.06 ml/L), severe behavioral abnormalities were recorded. These included loss of balance, uncoordinated and spiraling movements accompanied by jerks, and increased frequency of gasping at the water surface. At the highest concentration (0.06 ml/L), fish displayed extreme stress responses such as lateral recumbency at the bottom of the tank, rapid opercular movements with an open mouth, and eventual mortality.

These behavioral changes indicate progressive neurotoxic and respiratory distress effects induced by benzalkonium chloride exposure, with severity directly proportional to the concentration.

Table 2: Behavioral responses of Cyprinus carpio exposed to benzalkonium chloride

Concentration (ml/L)	Normal Swimming	Erratic Swimming & Gasping	Loss of Balance	Spiraling Movements	Lying at Bottom	Rapid Opercular Movement
0.01	Yes	No	No	No	No	No
0.02	Yes	Yes	No	No	No	No
0.03	No	Yes	Yes	No	No	No
0.04	No	Yes	Yes	Yes	No	No
0.05	No	Yes	Yes	Yes	Yes	No
0.06	No	Yes	Yes	Yes	Yes	Yes

Biochemical Studies

Glucose

Exposure to benzalkonium chloride caused a significant elevation in glucose levels in the tissues of *Cyprinus carpio*. In control fish, glucose levels were 0.131 mg/g in muscle and 0.085 mg/g in gills, whereas exposed fish exhibited increased levels of 0.168 mg/g and 0.108 mg/g, respectively. This increase reflects a stress-induced hyperglycemic condition associated with toxic exposure.

Tabel 3: Glucose content in tissues of Cyprinus carpio

Tissue	Control (mg/g)	BAC Exposed (mg/g)
Muscle	0.131	0.168
Gills	0.085	0.108

Glycogen

A pronounced depletion of glycogen reserves was observed in both tissues. In muscle, glycogen levels decreased from 0.125 mg/g to 0.015 mg/g, and in gills from 0.120 mg/g to 0.060 mg/g following exposure. This reduction indicates increased utilization of stored carbohydrates to meet elevated energy demands under stress conditions (Sousa *et al.,* 2023) [11].

Tabel 4: Glycogen content in tissues of Cyprinus carpio

Tissue	Control (mg/g)	BAC Exposed (mg/g)
Muscle	0.125	0.015
Gills	0.120	0.060

Protein

Protein content was also significantly reduced in exposed fish. Muscle protein levels declined from 0.650 mg/g to 0.482 mg/g, while gill protein decreased from 0.870 mg/g to 0.749 mg/g. This suggests enhanced proteolysis and metabolic imbalance due to toxic stress (Zeng *et al.,* 2024) [17].

Tabel 5: Protein content in tissues of Cyprinus carpio

Tissue	Control (mg/g)	BAC Exposed (mg/g)
Muscle	0.650	0.482
Gills	0.870	0.749

Histological Changes

Gills

Gill tissues exhibited severe structural damage, including epithelial desquamation, necrosis (telangiectasia) of secondary lamellae, shrinkage, and hypertrophy and hyperplasia at the lamellar base. These changes indicate impaired respiratory efficiency and physiological stress.

Muscles

Muscle tissues showed marked alterations such as nuclear proliferation, irregular fiber size, degeneration and atrophy of muscle bundles, along with separation and thickening of fibers, indicating compromised structural integrity.

Discussion

The findings of this study clearly demonstrate that benzalkonium chloride induces significant toxic effects in *Cyprinus carpio*, affecting biochemical, behavioral, and histological parameters.

Biochemical Impacts

Glucose Levels

The elevation in glucose levels suggests activation of stress-response pathways, leading to increased glucose mobilization to meet energy demands. Similar stress-induced hyperglycemia has been reported in fish exposed to disinfectants and environmental pollutants (Zeng *et al.*, 2024) [17].

Glycogen Levels

The depletion of glycogen reserves indicates enhanced glycogenolysis, reflecting increased metabolic activity under stress conditions. Previous studies also report similar glycogen depletion in fish exposed to benzalkonium chloride (Sousa *et al.*, 2023) [11].

Protein Levels

The reduction in protein content may be due to increased protein catabolism, where proteins are utilized as an alternative energy source during stress. Such metabolic disturbances have been widely observed in toxicological studies involving aquatic organisms (Liu *et al*., 2024) [6].

Histopathological Effects

Gills

Severe damage to gill tissues, including epithelial erosion and necrosis, indicates impaired respiratory and osmoregulatory functions. These alterations are consistent with earlier studies reporting structural damage in fish exposed to BAC and similar toxicants (Mukai *et al.*, 2024) [7].

Muscles

Muscle tissue degeneration and atrophy suggest disruption of structural proteins and impaired locomotion. Such changes may reduce the survival capacity of fish in natural environments.

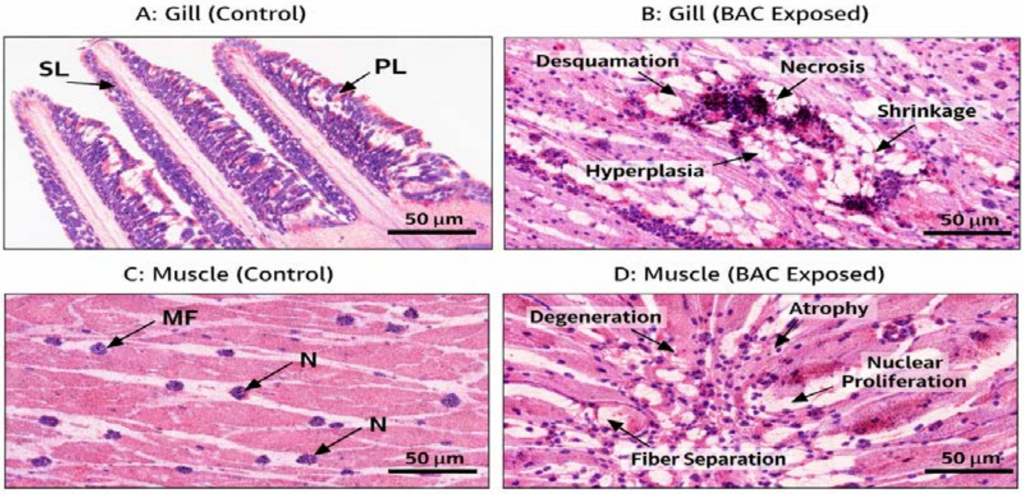

Figure 1. Histological changes in *Cyprinus carpio* tissues. (A) Gill of control fish showing normal structure: (B) Gill of BAC-exposed fish showing desquamation, necrosis, shrinkage, and hyperplasic; (C) Muscle of control fish with normal fibers; (D) Muscle of BAC-exposed fish showing degeneration, atrophy, and nuclear proliferation.

Behavioral Changes

Behavioral abnormalities such as erratic swimming, loss of equilibrium, and increased opercular movements are indicative of toxic stress and respiratory impairment. Experimental studies have shown that benzalkonium chloride exposure alters behavior and induces stress-related responses in fish (Sousa *et al.*, 2023) [11].

Conclusion

The present study demonstrates that benzalkonium chloride exerts significant toxic effects on *Cyprinus carpio*, even at relatively low concentrations. The observed increase in glucose levels, depletion of glycogen reserves, and reduction in protein content indicate severe metabolic disturbances. Histopathological findings further confirm extensive damage to gill and muscle tissues, which can impair respiration and movement.

Additionally, behavioral alterations observed during exposure reflect physiological stress and toxicity. Recent studies further support that benzalkonium chloride can affect immune function, metabolism, and reproductive health in aquatic organisms, highlighting its broader ecological impact (Zeng *et al.*, 2024; Mukai *et al.*, 2024) [7, 17].

Overall, these findings emphasize the ecological risks associated with benzalkonium chloride contamination and underline the need for strict environmental regulations, improved wastewater treatment, and responsible usage of disinfectants to safeguard aquatic ecosystems.

Recommendations for Future Research

1. Long-Term Exposure Studies

Future investigations should focus on chronic exposure experiments to better understand the long-term effects of benzalkonium chloride on fish health, growth, and reproductive performance.

2. Multi-Species Evaluation

Further studies are needed to assess the impact of benzalkonium chloride on a wider range of aquatic organisms, including different freshwater and marine species, to gain a comprehensive understanding of its ecological consequences.

3. Mechanistic and Molecular Studies

Research should be directed toward elucidating the underlying molecular and cellular mechanisms responsible for benzalkonium chloride-induced stress, toxicity, and tissue damage, which may help in developing effective mitigation strategies.

4. Environmental Monitoring Programs

Regular monitoring of aquatic ecosystems should be implemented to detect and quantify benzalkonium chloride and similar contaminants, ensuring early identification of pollution and protection of aquatic biodiversity.

5. Biomarker Development

Future work may focus on identifying sensitive biochemical and molecular biomarkers that can serve as early warning indicators of benzalkonium chloride toxicity in aquatic organisms.

6. Wastewater Treatment Strategies

There is a need to explore advanced and efficient wastewater treatment technologies capable of removing benzalkonium chloride and related compounds before their release into natural water bodies.

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