

# Multiorgan histopathological alterations in grass carp (*Ctenopharyngodon idella*) following azoxystrobin exposure

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## ABSTRACT

Azoxystrobin, a strobilurin fungicide widely used in agriculture, is increasingly detected in freshwater ecosystems, raising concerns about its potential impact on non-target aquatic species such as grass carp (*Ctenopharyngodon idella*). This study focused exclusively on the histopathological alterations induced by azoxystrobin (25% SC; a commercial suspension concentrate formulation) in the gills, liver, kidney, and intestine of *C. idella* under acute (1.6 ppm, 24 h) and short-term sublethal (0.16 ppm, 4–8 days) exposure conditions. Exposure produced clear concentration and time-dependent changes, including epithelial lifting and lamellar fusion in the gills, hepatocellular vacuolation and necrosis in the liver, tubular degeneration in the kidney, and villus atrophy and mucosal ulceration in the intestine. Lesion severity increased with exposure duration and concentration, indicating organ-specific structural impairments. The histopathological alterations observed in this study complement previously reported biochemical and behavioral disturbances caused by azoxystrobin exposure in freshwater fish. The use of a water-dispersible commercial formulation eliminated the need for solvent or positive controls, thereby reducing animal use while maintaining experimental validity. Overall, the findings provide the first detailed multiorgan histopathological characterization of azoxystrobin toxicity in grass carp and highlight histopathology as a sensitive endpoint for evaluating pesticide effects in aquaculture-linked freshwater environments.

## 1. INTRODUCTION

Freshwater aquaculture systems are increasingly affected by agricultural runoff carrying persistent agrochemicals into natural and cultivated water. Among them, azoxystrobin, a broad-spectrum strobilurin fungicide, has gained attention because of its high stability, systemic activity, and frequent detection in aquatic environments. Although they are highly effective at controlling phytopathogens, their unintended impacts on non-target aquatic organisms, particularly fish, have raised increasing ecotoxicological concerns [1,2].

Grass carp (*Ctenopharyngodon idella*), a species of major aquaculture and ecological importance in Asia, is often reared in integrated rice–fish systems where pesticide exposure is common. Previous experiments in which azoxystrobin was applied to grass carp revealed toxicological results of hematological and biochemical biomarkers, oxidative and respiratory alterations, acute toxicity, and behavioral responses [3–5]. These investigations revealed oxidative stress, reduced oxygen consumption, and disruptions in blood parameters. However,

despite these findings, corresponding organ-level histopathological evidence that provides direct visualization of tissue damage has not been fully characterized for *C. idella* under controlled exposure regimes.

Histopathological analysis serves as a sensitive and widely accepted diagnostic tool in aquatic toxicology, enabling direct assessment of cellular and tissue-level injury in metabolically active organs such as the gills, liver, kidney, and intestine [6,7]. Lesions, including those associated with epithelial lifting, vacuolation, lamellar fusion, necrosis, and villous atrophy, act as reliable biomarkers of sublethal stress and are often correlated with functional impairment [8,9]. While many studies have reported similar histological disturbances in fish exposed to pesticides, others have described partial or reversible recovery following depuration or low-dose exposure [2,10], indicating that such effects may vary by species, exposure duration, and formulation.

Therefore, the present study focused exclusively on histopathological alterations in the gills, liver, kidney, and intestine of *C. idella* exposed to azoxystrobin (25% SC; a commercial suspension concentrate formulation) under acute and sublethal exposure conditions. These concentrations were derived from previously established 96 h lethal concentration (LC<sub>50</sub>) data (11 mg/L) for azoxystrobin in *C. idella* [3,4] and represent ecotoxicologically relevant fractions of the LC<sub>50</sub> value, which was selected to induce measurable but non-lethal tissue responses. The specific objectives were to (i) characterize organ-specific lesions, (ii) assess their progression and severity across exposure regimens, and

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(iii) compare the observed alterations with previously reported pesticide-induced pathologies in freshwater teleosts. By emphasizing histological evidence, this work complements our earlier biochemical and behavioral findings and contributes to a more complete understanding of azoxystrobin toxicity in aquaculture-relevant fish species.

## 2. MATERIALS AND METHODS

### 2.1. Experimental Fish and Acclimatization

Juvenile *C. idella* (grass carp; mean weight =  $7.12 \pm 0.54$  g, mean total length =  $6.45 \pm 0.28$  cm;  $n = 150$ ) were procured from a certified aquaculture hatchery in Visakhapatnam District, Andhra Pradesh, India. The fish were transported in oxygenated containers and acclimatized for 15 days in 300-L aerated fiberglass tanks containing dechlorinated tap water. The tap water was dechlorinated by aeration for 48 h before use, and residual chlorine levels were confirmed to be below detectable limits ( $<0.01$  mg/L) through a portable chlorine test kit [111]. The water temperature was maintained at  $27 \pm 1.5^\circ\text{C}$ , the pH was 7.2–7.5, and the dissolved oxygen concentration was  $\geq 6.5$  mg/L under a 12 h light/12 h dark photoperiod. The fish were fed a commercial pelleted diet (35% crude protein; Growel Feeds Pvt. Ltd., India) at 2% of body weight daily. The proximate composition of the feed (on a dry weight basis) is presented in Table 1. Feeding was withheld 24 h before exposure. No mortality or abnormal behavior was observed during acclimatization.

### 2.2. Test Chemical

A commercial azoxystrobin suspension concentrate (25% SC; purity  $\geq 98\%$ ) was used. The formulation was water-dispersible, and test concentrations were prepared through direct dilution in dechlorinated tap water without the use of organic solvents or carriers. According to the Organisation for Economic Co-operation and Development (OECD) guideline 203 and OECD guidance no. 23, solvent control is not required when the test substance is directly soluble in water [12,13].

The exposure medium consisted of continuously aerated dechlorinated tap water maintained under static-renewal conditions. Water quality parameters were monitored daily and maintained within optimal ranges for *C. idella*: temperature  $26 \pm 1^\circ\text{C}$ , pH  $7.2 \pm 0.3$ , dissolved oxygen  $6.8 \pm 0.4$  mg/L, total hardness  $120 \pm 10$  mg  $\text{CaCO}_3/\text{L}$ , and total ammonia  $<0.02$  mg/L. The exposure media were renewed every 24 h to maintain concentration stability and overall water quality.

Although commercial formulations contain surfactants and adjuvants ( $\sim 70$ – $77\%$  inert ingredients), they are directly water dispersible. Therefore, neither a solvent nor a positive control group was included. This choice aligns with OECD and CPCSEA ethical standards to minimize animal use and avoid redundant treatments.

**Table 1:** Proximate composition of the commercial feed used for *Ctenopharyngodon idella*.

| Parameter                   | Composition(% dry weight basis) | Analytical method reference |
|-----------------------------|---------------------------------|-----------------------------|
| Crude protein               | 35.00 $\pm$ 0.42                | AOAC (2016) [31]            |
| Crude lipid                 | 6.20 $\pm$ 0.18                 | AOAC (2016) [31]            |
| Crude fiber                 | 5.10 $\pm$ 0.22                 | AOAC (2016) [31]            |
| Ash                         | 8.75 $\pm$ 0.15                 | AOAC (2016) [31]            |
| Moisture                    | 9.80 $\pm$ 0.25                 | AOAC (2016) [31]            |
| Nitrogen-free extract (NFE) | 35.15 $\pm$ 0.30                | Calculated by difference    |

Values represent the means $\pm$ SDs ( $n=3$ ). The diet was a floating, extruded commercial carp feed produced by Growel Feeds Pvt. Ltd., Andhra Pradesh, India.

### 2.3. Experimental Design

The fish were randomly distributed into the following groups, each in triplicate 100-L aquaria (15 fish/replicate;  $n = 45/\text{group}$ ):

- Control: Pesticide-free water.
- Sublethal exposure: 0.16 ppm for 4 and 8 days.
- Acute exposure: 1.6 ppm for 24 h.

The concentrations were determined on the basis of preliminary acute toxicity tests, which yielded a 96 h  $\text{LC}_{50}$  value of 11 mg/L for azoxystrobin in *C. idella* (Finney, 1971) [14]. In accordance with OECD guideline 203 and ecological risk assessment principles [12], the acute exposure concentration (1.6 ppm) corresponded to approximately  $1/7^{\text{th}}$  of the  $\text{LC}_{50}$ , whereas the sub  $\text{LC}_{50}$  (0.16 ppm) represented  $1/70^{\text{th}}$  of the  $\text{LC}_{50}$ . These values lie within the ecotoxicologically relevant range reported for strobilurin fungicides in freshwater [15,16].

Exposure durations were chosen to represent short-term acute exposure (24 h) and extended sublethal exposure (4–8 days), which is consistent with aquatic toxicology protocols for distinguishing immediate versus progressive histopathological responses.

The water in all tanks was renewed every 24 h to ensure exposure uniformity. Details of the concentration, exposure duration, and replicates used for each treatment group are provided in Table 2. Since azoxystrobin (25% SC) is water dispersible, no solvent control was used. Positive-control toxicants (e.g., copper sulfate) were not included to reduce unnecessary fish use, and because OECD guidance no. 23 (2019) [13] states that they are optional unless specifically required for reference purposes.

### 2.4. Determination of the 96 h $\text{LC}_{50}$ (Regression Analysis)

The 96 h median  $\text{LC}_{50}$  of azoxystrobin (25% SC) for *C. idella* was determined through probit regression analysis (Finney, 1971) [14] through mortality data from range-finding tests. A binomial generalized linear model with a probit link was applied to mortality proportions against  $\log_{10}$ -transformed concentrations. The calculated  $\text{LC}_{50}$  was 11 mg/L (95% CI: 9.8–12.4 mg/L). This  $\text{LC}_{50}$  value guided the selection of acute (1.6 ppm  $\approx 1/7 \text{ LC}_{50}$ ) and sublethal (0.16 ppm  $\approx 1/70 \text{ LC}_{50}$ ) test concentrations.

### 2.5. Tissue Sampling and Processing

At each exposure endpoint, five fish per replicate ( $n = 15$  per group) were randomly selected and euthanized through buffered MS-222 (100 mg/L). The gills, liver, kidney, and intestine were excised, rinsed in 0.75% saline, and fixed in aqueous Bouin's solution for 24–48 h. Tissues were processed through standard histological methods [17,18], dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin wax. Sections (5–6  $\mu\text{m}$ ) were cut through a rotary microtome and mounted on poly-L-lysine-coated slides for staining.

### 2.6. Staining and Microscopy

Slides were stained with Ehrlich's hematoxylin and eosin (H&E) following Humason (1972) [19]. Photomicrographs were captured through an Olympus BX53 microscope fitted with a DP74 digital camera at 40 $\times$  and 100 $\times$  magnifications.

### 2.7. Lesion Scoring

Histopathological alterations were graded through a semiquantitative four-point scale: 0 = Absent, 1 = Mild, 2 = Moderate, and 3 = Severe. For each fish, three tissue sections per organ and five random microscopic

fields per section were evaluated. The mean lesion scores  $\pm$  standard deviations were calculated for each lesion type and treatment group.

The major lesion categories included epithelial lifting, lamellar fusion, hepatocyte vacuolation, tubular degeneration, and villous atrophy. The summarized lesion scores, corresponding  $P$  values, and effect sizes ( $\eta^2$ ) are presented in Tables 3-6. Within each lesion type, means followed by different superscript letters (a-d or a-e) denote significant differences ( $P < 0.05$ ) according to one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) test.

## 2.8. Quality Assurance and Ethical Approval

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Department of Zoology and Aquaculture, Acharya Nagarjuna University, Andhra Pradesh, India (Approval No. ANUCS/IAEC/AH/Z/14/2024). All procedures complied with the guidelines of the Committee for the Purpose of Control and Supervision of

Experiments on Animals (CPCSEA), Government of India. The study adhered to the National Guidelines for the Care and Use of Laboratory Animals and followed the ARRIVE reporting standards.

All experimental procedures were conducted in accordance with OECD guideline 203 (fish acute toxicity test) [12] and established recommendations for histopathological evaluation in aquatic toxicology. The fish were handled and euthanized humanely through buffered MS-222 (100 mg/L) to minimize distress. Lesion scoring was independently verified by two observers to minimize subjective bias.

## 2.9. Statistical Analysis

All the data were tested for normality (Shapiro–Wilk) and homogeneity of variance (Levene's test). One-way ANOVA followed by Tukey's HSD *post hoc* comparisons was applied to determine significant differences among groups ( $P < 0.05$ ). Effect sizes ( $\eta^2$ ) were calculated to evaluate treatment magnitude. Statistical analyses were performed through Python (v3.10) with SciPy and Statsmodels libraries in a Jupyter

**Table 2:** Experimental design detailing the azoxystrobin (25% SC) concentration, exposure duration, toxicological purpose, and number of replicates used for each treatment group in *Ctenopharyngodon idella*.

| Group        | Azoxystrobin (25% SC) concentration (ppm) | Exposure duration | Toxicological purpose               | Number of replicates |
|--------------|---|-------------------|-------------------------------------|----------------------|
| Control      | 0   | 8 days            | Baseline/reference group            | 3                    |
| Sublethal-4d | 0.16                                      | 4 days            | sublethal assessment                | 3                    |
| Sublethal-8d | 0.16                                      | 8 days            | Chronic sublethal exposure study    | 3                    |
| Lethal-24 h  | 1.6                                       | 24 h              | Acute lethal exposure investigation | 3                    |

Concentrations were selected on the basis of preliminary  $LC_{50}$  determination for azoxystrobin and corroborated with existing ecotoxicological studies [3].

**Table 3:** Mean lesion scores ( $\pm$ SD) for gill histopathological alterations in *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC) ( $n=5$  fish per group, three sections per fish).

| Lesion type              | Control                      | Sublethal 4 d                | Sublethal 8 d                | Lethal 24 h                  | $P$ value | $\eta^2$ |
|--------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-----------|----------|
| Epithelial lifting       | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.75 $\pm$ 0.14 <sup>b</sup> | 1.40 $\pm$ 0.25 <sup>c</sup> | 2.10 $\pm$ 0.21 <sup>d</sup> | <0.001    | 0.84     |
| Lamellar fusion          | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.85 $\pm$ 0.16 <sup>b</sup> | 1.62 $\pm$ 0.22 <sup>c</sup> | 2.20 $\pm$ 0.18 <sup>d</sup> | <0.001    | 0.85     |
| Vacuolation              | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.60 $\pm$ 0.11 <sup>b</sup> | 1.15 $\pm$ 0.21 <sup>c</sup> | 1.85 $\pm$ 0.23 <sup>d</sup> | <0.001    | 0.82     |
| Hyperplasia              | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.45 $\pm$ 0.10 <sup>b</sup> | 0.98 $\pm$ 0.19 <sup>c</sup> | 1.65 $\pm$ 0.27 <sup>d</sup> | <0.001    | 0.81     |
| Vascular congestion      | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.88 $\pm$ 0.13 <sup>b</sup> | 1.25 $\pm$ 0.17 <sup>c</sup> | 1.90 $\pm$ 0.20 <sup>d</sup> | <0.001    | 0.83     |
| Necrosis of lamellae     | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.62 $\pm$ 0.12 <sup>b</sup> | 1.35 $\pm$ 0.22 <sup>c</sup> | 1.80 $\pm$ 0.24 <sup>d</sup> | <0.001    | 0.84     |
| Pillar cell degeneration | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.35 $\pm$ 0.09 <sup>b</sup> | 0.84 $\pm$ 0.18 <sup>c</sup> | 1.45 $\pm$ 0.20 <sup>d</sup> | <0.001    | 0.82     |
| Interlamellar edema      | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.50 $\pm$ 0.12 <sup>b</sup> | 0.96 $\pm$ 0.17 <sup>c</sup> | 1.70 $\pm$ 0.26 <sup>d</sup> | <0.001    | 0.83     |

Values represent the means $\pm$ SDs ( $n=5$ ). Within each row, means followed by different superscript letters (a-d) are significantly different ( $P<0.05$ ) according to one-way ANOVA, followed by Tukey's *post hoc* test. Effect sizes ( $\eta^2$ ) indicate large effects ( $>0.8$ ) for all lesions.

**Table 4:** Mean lesion scores ( $\pm$ SD) for liver histopathological alterations in *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC).

| Lesion type                     | Control                      | Sublethal 4 d                | Sublethal 8 d                | Lethal 24 h                  | $P$ value | $\eta^2$ |
|---------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-----------|----------|
| Hepatocyte vacuolation          | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.10 $\pm$ 0.18 <sup>b</sup> | 1.80 $\pm$ 0.20 <sup>c</sup> | 2.05 $\pm$ 0.21 <sup>d</sup> | <0.001    | 0.86     |
| Nuclear condensation (pyknosis) | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.65 $\pm$ 0.12 <sup>b</sup> | 1.32 $\pm$ 0.22 <sup>c</sup> | 1.65 $\pm$ 0.18 <sup>d</sup> | <0.001    | 0.84     |
| Cytoplasmic degeneration        | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.02 $\pm$ 0.14 <sup>b</sup> | 1.75 $\pm$ 0.19 <sup>c</sup> | 2.10 $\pm$ 0.23 <sup>d</sup> | <0.001    | 0.86     |
| Hepatic cord disruption         | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.78 $\pm$ 0.10 <sup>b</sup> | 1.48 $\pm$ 0.21 <sup>c</sup> | 1.88 $\pm$ 0.19 <sup>d</sup> | <0.001    | 0.85     |
| Sinusoidal dilation             | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.95 $\pm$ 0.11 <sup>b</sup> | 1.42 $\pm$ 0.17 <sup>c</sup> | 1.85 $\pm$ 0.20 <sup>d</sup> | <0.001    | 0.84     |
| Coagulative necrosis            | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.55 $\pm$ 0.09 <sup>b</sup> | 1.36 $\pm$ 0.20 <sup>c</sup> | 1.91 $\pm$ 0.24 <sup>d</sup> | <0.001    | 0.85     |
| Hemorrhage                      | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.45 $\pm$ 0.08 <sup>b</sup> | 1.28 $\pm$ 0.18 <sup>c</sup> | 1.72 $\pm$ 0.21 <sup>d</sup> | <0.001    | 0.82     |
| Bile retention (cholestasis)    | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.30 $\pm$ 0.06 <sup>b</sup> | 0.72 $\pm$ 0.10 <sup>c</sup> | 1.21 $\pm$ 0.13 <sup>d</sup> | <0.001    | 0.81     |

Values represent the means $\pm$ SDs ( $n=5$ ). Within each row, means followed by different superscript letters (a-d) differ significantly ( $P<0.05$ ) according to one-way ANOVA followed by Tukey's HSD test. Effect sizes ( $\eta^2$ ) indicate large effects ( $>0.8$ ) for all lesions.

**Table 5:** Mean lesion scores ( $\pm$ SD) for kidney histopathological changes in *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC).

| Lesion                         | Control                      | Sublethal (4 days)           | Sublethal (8 days)           | Lethal (24 h)                | P value | $\eta^2$ |
|--------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|---------|----------|
| Tubular degeneration           | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.25 $\pm$ 0.50 <sup>b</sup> | 2.20 $\pm$ 0.40 <sup>c</sup> | 3.10 $\pm$ 0.30 <sup>d</sup> | <0.001  | 0.88     |
| Cytoplasmic vacuolation        | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.30 $\pm$ 0.47 <sup>b</sup> | 2.40 $\pm$ 0.49 <sup>c</sup> | 2.85 $\pm$ 0.38 <sup>d</sup> | <0.001  | 0.86     |
| Glomerular atrophy             | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.65 $\pm$ 0.24 <sup>b</sup> | 1.10 $\pm$ 0.28 <sup>c</sup> | 2.05 $\pm$ 0.34 <sup>d</sup> | <0.001  | 0.84     |
| Bowman's space widening        | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.75 $\pm$ 0.21 <sup>b</sup> | 1.25 $\pm$ 0.33 <sup>c</sup> | 2.30 $\pm$ 0.36 <sup>d</sup> | <0.001  | 0.85     |
| Cloudy swelling                | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.10 $\pm$ 0.28 <sup>b</sup> | 1.90 $\pm$ 0.44 <sup>c</sup> | 2.00 $\pm$ 0.30 <sup>d</sup> | <0.001  | 0.83     |
| Tubule structure loss          | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.70 $\pm$ 0.23 <sup>b</sup> | 1.35 $\pm$ 0.39 <sup>c</sup> | 2.10 $\pm$ 0.37 <sup>d</sup> | <0.001  | 0.84     |
| Interstitial cell infiltration | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.55 $\pm$ 0.22 <sup>b</sup> | 1.00 $\pm$ 0.27 <sup>c</sup> | 1.30 $\pm$ 0.29 <sup>d</sup> | <0.001  | 0.80     |

Values represent the means $\pm$ SDs (n=5). Within each row, means followed by different superscript letters (a–d) differ significantly ( $P < 0.05$ ) according to one-way ANOVA, followed by Tukey's HSD test. Effect sizes ( $\eta^2$ ) indicate large effects ( $>0.8$ ) for all lesions.

**Table 6:** Mean lesion scores ( $\pm$ SD) for intestinal histopathological changes in *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC).

| Lesion type                  | Control                      | Sublethal 24 h               | Lethal 24 h                  | Sublethal 4 d                | Sublethal 8 d                | P value | $\eta^2$ |
|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|---------|----------|
| Villus atrophy               | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.85 $\pm$ 0.10 <sup>b</sup> | 1.75 $\pm$ 0.12 <sup>c</sup> | 2.15 $\pm$ 0.13 <sup>d</sup> | 2.90 $\pm$ 0.10 <sup>e</sup> | <0.001  | 0.89     |
| Epithelial lifting           | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.00 $\pm$ 0.14 <sup>b</sup> | 2.10 $\pm$ 0.11 <sup>c</sup> | 2.30 $\pm$ 0.12 <sup>d</sup> | 2.95 $\pm$ 0.08 <sup>e</sup> | <0.001  | 0.88     |
| Goblet cell alterations      | 0.20 $\pm$ 0.05 <sup>a</sup> | 1.60 $\pm$ 0.11 <sup>b</sup> | 1.10 $\pm$ 0.10 <sup>b</sup> | 2.20 $\pm$ 0.09 <sup>c</sup> | 2.85 $\pm$ 0.07 <sup>d</sup> | <0.001  | 0.87     |
| Vacuolar degeneration        | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.10 $\pm$ 0.12 <sup>b</sup> | 1.80 $\pm$ 0.14 <sup>c</sup> | 2.10 $\pm$ 0.11 <sup>d</sup> | 2.95 $\pm$ 0.05 <sup>e</sup> | <0.001  | 0.88     |
| Leukocyte infiltration       | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.20 $\pm$ 0.05 <sup>b</sup> | 1.10 $\pm$ 0.12 <sup>c</sup> | 2.25 $\pm$ 0.10 <sup>d</sup> | 3.00 $\pm$ 0.00 <sup>e</sup> | <0.001  | 0.89     |
| Mucosal ulceration           | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.15 $\pm$ 0.06 <sup>b</sup> | 1.20 $\pm$ 0.10 <sup>c</sup> | 1.85 $\pm$ 0.09 <sup>d</sup> | 3.00 $\pm$ 0.00 <sup>e</sup> | <0.001  | 0.88     |
| Congestion in lamina propria | 0.00 $\pm$ 0.00 <sup>a</sup> | 1.00 $\pm$ 0.10 <sup>b</sup> | 1.90 $\pm$ 0.08 <sup>c</sup> | 2.40 $\pm$ 0.10 <sup>d</sup> | 3.00 $\pm$ 0.00 <sup>e</sup> | <0.001  | 0.89     |
| Muscularis degeneration      | 0.00 $\pm$ 0.00 <sup>a</sup> | 0.25 $\pm$ 0.08 <sup>b</sup> | 1.00 $\pm$ 0.11 <sup>c</sup> | 2.10 $\pm$ 0.12 <sup>d</sup> | 2.95 $\pm$ 0.06 <sup>e</sup> | <0.001  | 0.87     |

Values represent the means $\pm$ SDs (n=5). Within each row, means followed by different superscript letters (a–e) differ significantly ( $P < 0.05$ ) on the basis of one-way ANOVA and Tukey's HSD test.

Notebook environment (Anaconda distribution). Superscript letters in Tables 3-6 denote statistically significant group differences ( $P < 0.05$ ).

## 2.10. Correlation Analysis of Lesion Scores

Spearman's rank correlation analysis was performed to evaluate intra-organ relationships among lesion types in the gill, liver, kidney, and intestinal tissues [Tables 3-6]. Correlation matrices were visualized as heatmaps [Figure 1] through GraphPad Prism (version 10.0; GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at  $P < 0.05$ .

## 2.11. Principal Component Analysis (PCA)

PCA was performed on standardized mean lesion score data from the gill, liver, kidney, and intestinal tissues to identify major variables explaining exposure-dependent variance. The analysis was conducted in R (version 4.x) through the *prcomp* function with unit variance scaling. PCA biplots were generated to visualize treatment clustering and lesion loadings [Figure 2].

## 3. RESULTS AND DISCUSSION

### 3.1. Gill Histopathology

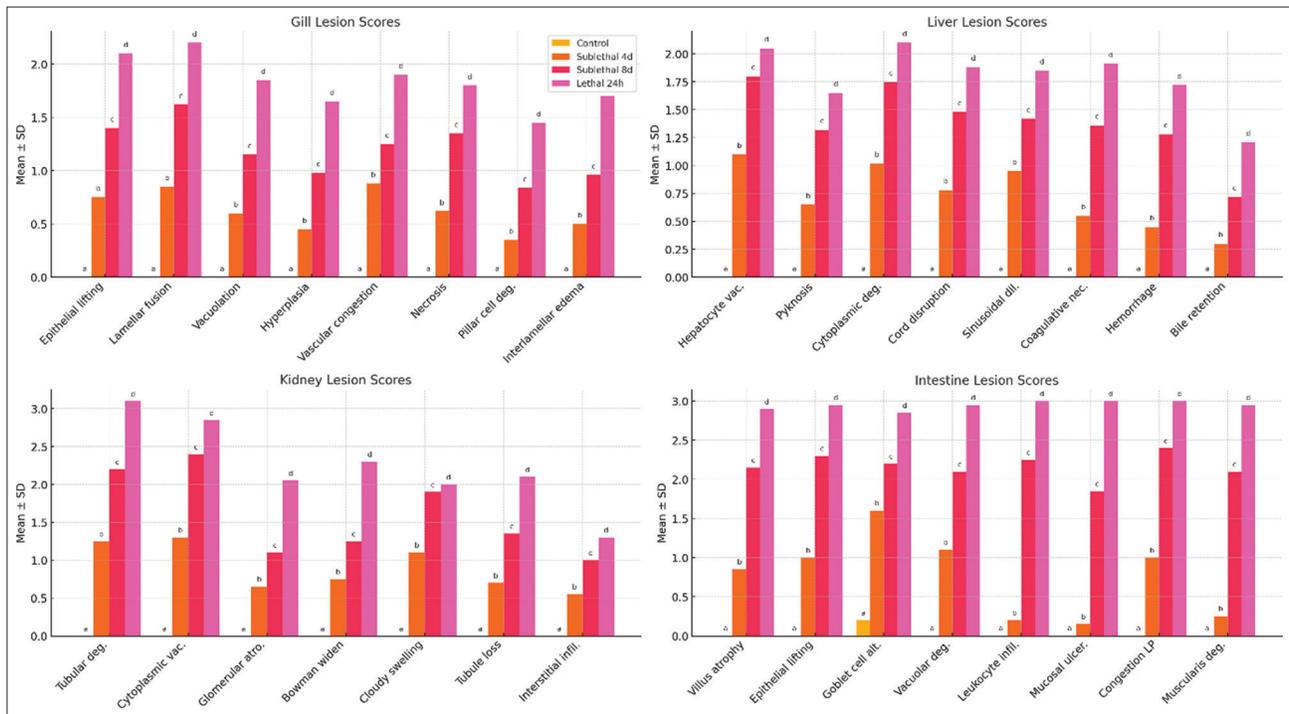
The control fish presented normal primary and secondary lamellae with intact epithelial and vascular organization [Plate Ia - G1]. Azoxystrobin exposure caused distinct, concentration, and time-dependent gill alterations. After 4 days of sublethal exposure (0.16 ppm), mild epithelial lifting and vacuolation were observed, indicating early adaptive responses to reduce toxicant diffusion. By day 8, the lesions intensified to include lamellar fusion, vascular congestion, and pillar-cell degeneration, suggesting progressive structural impairment.

Acute exposure (1.6 ppm, 24 h) induced severe disruptions, such as hemorrhage, epithelial necrosis, and complete lamellar disorganization [Plate Ib - G2].

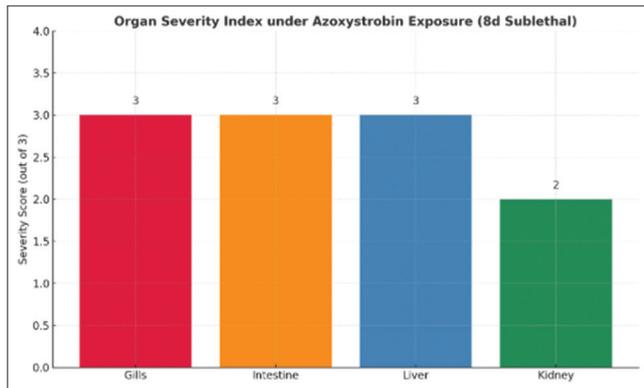
The lesion scores [Table 3] were significantly different ( $P < 0.001$ ), with the highest severity in the acute group. One-way ANOVA visualization [Figure 3] clearly revealed a dose-dependent increase in lesion severity. These lesions can be attributed to the direct toxic effects of azoxystrobin on gill epithelial and endothelial cells. Mechanistically, azoxystrobin inhibits mitochondrial electron transport at the Qo site of the cytochrome *bc<sub>1</sub>* complex (complex III), reducing ATP synthesis and increasing reactive oxygen species (ROS) generation [20]. Elevated ROS causes oxidative stress, lipid peroxidation, and membrane disruption, which compromise pillar-cell integrity and capillary function, leading to hemorrhage and necrosis. The resulting collapse of respiratory surfaces impairs oxygen exchange and induces hypoxia [15,21]. Comparable gill lesions have been reported in fish exposed to pyrethroids [22] and glyphosate [23]. Early epithelial lifting thus represents a protective barrier, whereas advanced necrosis denotes irreversible oxidative damage. Nazir *et al.* [10] reported partial recovery of gill architecture in *Cyprinus carpio* after depuration, suggesting that lesion reversibility depends on species resilience and exposure duration.

### 3.2. Liver Histopathology

Livers of control fish displayed polygonal hepatocytes arranged in cords with centrally located nuclei and intact sinusoids [Plate IIa]. After 4 days of sublethal exposure, mild hepatocyte vacuolation and cytoplasmic disorganization were noted, whereas 8 days of exposure led to marked vacuolation, nuclear pyknosis, and sinusoidal dilation. Acute exposure caused coagulative necrosis, hepatic-cord disruption, and bile retention [Plate IIb].

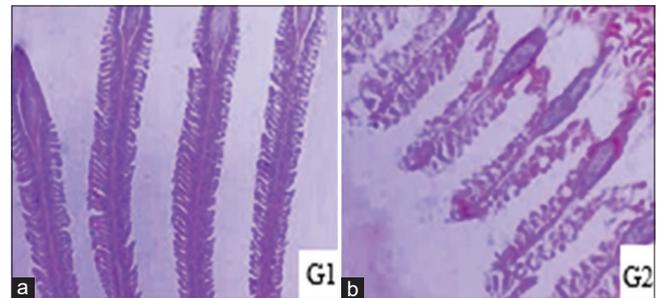


**Figure 1:** One-way analysis of variance (ANOVA) visualization of lesion severity across organs of *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC). Bar plots illustrate the mean ( $\pm$ SD) lesion severity scores in the gills, liver, kidney, and intestine of *C. idella* under control, sublethal (4 d, 8 d), and acute (24 h) azoxystrobin exposure conditions. The distinct superscript letters (a-d) indicate statistically significant differences ( $P < 0.05$ ) according to one-way ANOVA followed by Tukey's HSD test. Note: Data derived from Tables 2-5; plotted through Python (Matplotlib and Seaborn).



**Figure 2:** Organ severity index representing cumulative histopathological damage in *Ctenopharyngodon idella* after 8-day sublethal exposure to azoxystrobin (25% SC). The gill, liver, and intestine scores were the most severe (3/3), indicating extensive histological damage, such as lamellar fusion, villous erosion, and hepatic necrosis. The kidney was slightly less severe (2/3), showing notable but comparatively less extensive tubular and glomerular alterations.

These progressive lesions ([Table 4], also see [Figure 3] for comparative visualization) reflect hepatocellular stress and impaired metabolic function. Mechanistically, azoxystrobin-induced mitochondrial dysfunction reduces oxidative phosphorylation and elevates ROS levels in hepatocytes, triggering lipid peroxidation and vacuolar degeneration [16]. Prolonged oxidative damage disrupts membrane integrity and causes leakage of lysosomal enzymes, resulting in necrosis and bile stasis. Similar hepatic alterations have been reported



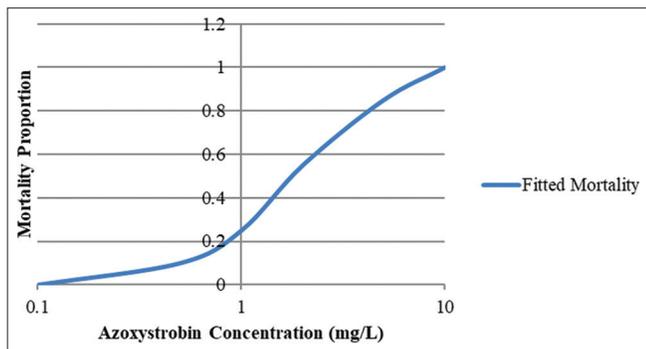
**Plate 1 (Gill):** Histological sections of gills from *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC). (a) G1 - Control showing intact lamellae with a normal epithelial lining and vascular integrity (100 $\times$ ). (b) G2) Sublethal group (4 days) showing epithelial lifting, vascular congestion, and early lamellar fusion (100 $\times$ ). H&E staining.

in *Clarias batrachus* under organophosphate exposure [24] and in *Labeo rohita* exposed to difenoconazole [25]. The progression from reversible vacuolation (4 days) to irreversible necrosis (8 days and acute) suggests that azoxystrobin overwhelms antioxidant defenses with prolonged exposure.

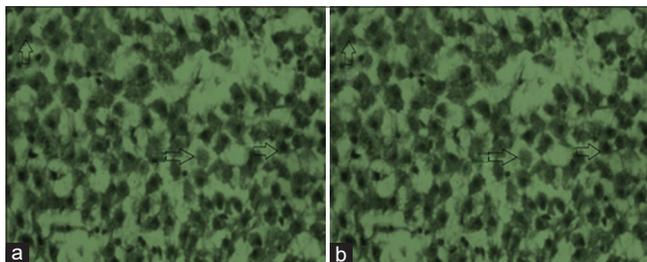
### 3.3. Kidney Histopathology

The kidneys of the control fish presented normal glomeruli and an intact tubular architecture [Plate IIIa]. After 4 days of sublethal exposure, mild tubular dilation and widening of Bowman's space were noted. By 8 days, cytoplasmic vacuolation and partial tubular necrosis appeared, whereas acute exposure produced severe tubular degeneration, hemorrhage, and inflammatory infiltration [Plate IIIb].

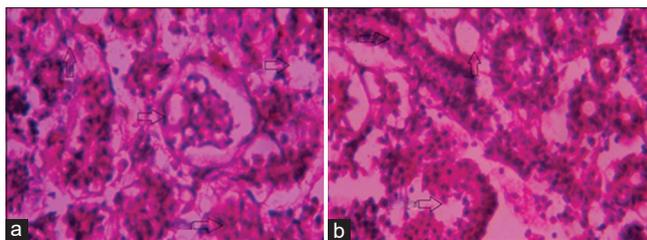
The lesion scores ([Table 5], also see [Figure 3] for comparative visualization) increased significantly with exposure time and concentration ( $P < 0.001$ ). These alterations suggest that azoxystrobin impairs renal osmoregulatory and excretory functions. The observed tubular degeneration is likely linked to oxidative stress-mediated mitochondrial injury and the disruption of ion transport proteins in renal epithelial cells. ROS-induced lipid peroxidation may also compromise tubular membrane integrity, causing cell lysis and glomerular collapse [10,26]. Similar renal pathology has been reported



**Figure 3:** Dose–response curve of azoxystrobin in *Ctenopharyngodon idella*. The fitted probit regression model describes the relationship between azoxystrobin concentration (mg/L) and observed mortality proportion in *C. idella*. The  $LC_{50}$  value was estimated from this model, showing a concentration-dependent increase in mortality. The curve demonstrates a good model fit ( $R^2 > 0.95$ ).



**Plate II (Liver):** Histological sections of livers from *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC). (a) Control showing normal hepatic cords (HC) and centrally arranged sinusoids (S). (b) Sublethal exposure (8 days) results in cytoplasmic vacuolation (CV), nuclear pyknosis (PN), disrupted hepatic cords (HCs), and dilated sinusoids (DSs). H&E staining, 40× magnification.



**Plate III (Kidney):** Kidney histology of *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC). (a) Control fish showing normal renal structure with intact glomeruli and proximal (PCT) and distal convoluted tubules (DCT). (b) Sublethal exposure (8 days) results in tubular epithelial degeneration, cytoplasmic vacuolation, cloudy swelling, glomerular shrinkage, and interstitial inflammation. Hematoxylin and eosin staining, magnification: 40×.

in fish exposed to heavy metals and pesticides, indicating a common oxidative mechanism. The partial regeneration observed in some species after depuration [10] implies that mild renal lesions may be reversible if oxidative stress is transient.

### 3.4. Intestinal Histopathology

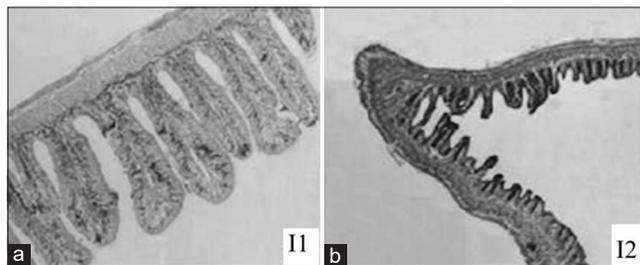
The intestines of the control fish presented long, slender villi with intact columnar epithelium and well-distributed goblet cells [Plate IVa - I1]. After 4 days of sublethal exposure, epithelial lifting, vacuolation, and reduced goblet-cell density were evident. By 8 days, villous atrophy, mucosal ulceration, and epithelial sloughing were prominent, indicating progressive mucosal injury. Acute exposure caused villous blunting, hemorrhage, and complete mucosal detachment [Plate IVb - I2].

The lesion scores ([Table 6], also see [Figure 3] for comparative visualization) significantly increased ( $P < 0.001$ ) with exposure time and concentration. Azoxystrobin likely disrupts intestinal integrity by inducing oxidative damage to enterocytes and reducing mucosal antioxidant enzyme activity, leading to epithelial apoptosis and compromised absorption. The decline in goblet cells suggests impaired mucus secretion, weakening the protective barrier and promoting inflammation [27,28]. Guo *et al.* (2024) [2] reported partial recovery at low azoxystrobin levels, implying that intestinal injury may be reversible under environmentally realistic conditions.

### 3.5. Integrative and Mechanistic Interpretation

Cross-organ comparisons revealed that the gills and intestines were the most sensitive to azoxystrobin, followed by the liver and kidney [Figure 4, Tables 7 and 8]. The organ-severity indices mirrored functional dependencies: gills for respiration, liver for detoxification, kidney for osmoregulation, and intestine for absorption. The pronounced gill and hepatic damage indicate that azoxystrobin primarily affects tissues with high oxygen demand and metabolic activity. A comparative overview of the cumulative histopathological lesion distribution among major organs is further visualized in Figure 5, which shows the trend of gills > intestine > liver > kidney.

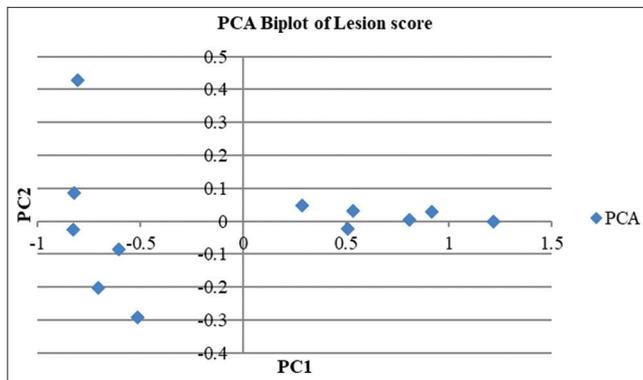
Mechanistically, azoxystrobin inhibits mitochondrial electron transport (Complex III), resulting in energy deprivation and ROS accumulation across tissues [16,20]. The generated oxidative stress leads to membrane lipid peroxidation, protein denaturation, and apoptosis, accounting for the vacuolation, necrosis, and epithelial degeneration observed histologically. Furthermore, formulation surfactants in the 25% SC product can enhance chemical uptake and



**Plate IV (Intestine):** Intestine histology of *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC). (a) (I1) Control group showing long, slender villi lined with intact columnar epithelium and normal goblet cell distribution. (b) (I2) Sublethal exposure (4 days) results in villus atrophy, goblet cell depletion, epithelial sloughing, and lamina propria congestion with early leukocytic infiltration. Hematoxylin and eosin staining, magnification: 40×.

compromise membrane integrity, amplifying toxicity beyond that of pure azoxystrobin [12,28].

The lesion scores increased significantly with both exposure duration (4 vs. 8 days) and concentration (0.16 vs. 1.6 ppm), confirming a robust exposure–response pattern ( $P < 0.001$ ;  $\eta^2 > 0.8$ ) [Tables 3-6]. Statistical visualization through one-way ANOVA further highlighted these significant differences among the treatments [Figure 3]. The regression model ( $y = -3.412 + 5.27 \log C$ ) yielded an  $LC_{50}$  of 11 mg/L (95% CI: 9.8–12.4), which is consistent with prior findings on strobilurin toxicity in freshwater fish [Figure 1]. Multivariate principal



**Figure 4:** Principal component analysis (PCA) of dose-dependent lesion responses in *Ctenopharyngodon idella*. PCA biplot summarizing multivariate associations among histopathological lesions across the gills, liver, and kidney.

The first two components explain more than 85% of the total variance. The increasing separation of the treatment groups along PC1 reflects dose-dependent toxicity, whereas the lesion loading shows gill epithelial lifting, hepatic vacuolation, and renal tubular degeneration as key discriminating features.

component analysis (PCA) confirmed dose-dependent clustering and lesion loading, with gill epithelial lifting, hepatic vacuolation, and renal tubular degeneration emerging as the dominant contributors [Figure 2].

### 3.6. Integration with Previous Studies

The present histopathological results complement earlier biochemical and hematological findings in *C. idella* exposed to azoxystrobin [3-5]. Organ-wise lesion severities showed strong positive correlations [Table 7], indicating consistent pathological progression across organs under increasing azoxystrobin exposure. Lesion frequency and severity across gill, liver, kidney, intestine, and muscle showed a clear concentration- and time-dependent escalation in grass carp [Table 8]. The resulting organ-sensitivity ranking highlights gills and liver as the principal targets of azoxystrobin toxicity in this species [Table 9]. Those studies reported decreased RBC counts and hemoglobin levels, elevated leukocyte counts, and increased levels of oxidative stress markers, which are consistent with the gill necrosis, hepatic vacuolation, and renal degeneration observed here. Together, these findings establish that azoxystrobin induces systemic oxidative injury, leading to both functional and structural impairment [Table 10].

Comparable responses across teleost species, such as epithelial lifting and hepatic necrosis in *Danio rerio* [2] and *Oreochromis niloticus* [29], indicate that the observed mechanisms are conserved across freshwater fish.

### 3.7. Environmental Relevance and Limitations

Azoxystrobin residues in agricultural runoff range from sub  $\mu\text{g/L}$  to several tens of  $\mu\text{g/L}$  in surface waters [2,30]. The present concentrations (0.16 and 1.6 mg/L) represent high-end or pulse exposures that may occur during heavy rainfall or accidental discharge. Although

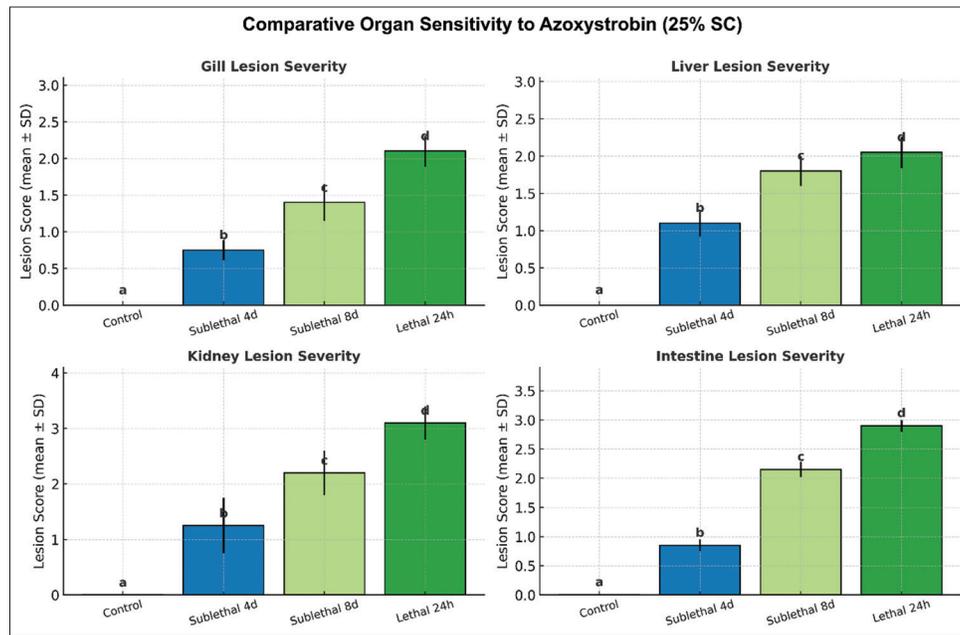
**Table 7:** Spearman correlations of organ-wise histopathological lesion severity in *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC).

|           | Gill                                       | Liver                                      | Kidney                                 | Intestine                               |
|-----------|--|--|--|---|
| Gill      | 1.0000<br>( $P=4.05 \times 10^{-201}$ )    | 0.9394<br>( $P=1.34 \times 10^{-1 \geq}$ ) | 0.9623<br>( $P=3.17 \times 10^{-16}$ ) | 0.8993<br>( $P=7.82 \times 10^{-11}$ )  |
| Liver     | 0.9394<br>( $P=1.34 \times 10^{-1 \geq}$ ) | 1.0000<br>( $P=4.05 \times 10^{-201}$ )    | 0.8898<br>( $P=2.40 \times 10^{-10}$ ) | 0.8779<br>( $P=8.42 \times 10^{-10}$ )  |
| Kidney    | 0.9623<br>( $P=3.17 \times 10^{-16}$ )     | 0.8898<br>( $P=2.40 \times 10^{-10}$ )     | 1.0000<br>( $P=0$ )                    | 0.8420<br>( $P=1.96 \times 10^{-8}$ )   |
| Intestine | 0.8993<br>( $P=7.82 \times 10^{-11}$ )     | 0.8779<br>( $P=8.42 \times 10^{-10}$ )     | 0.8420<br>( $P=1.96 \times 10^{-8}$ )  | 1.0000<br>( $P=4.05 \times 10^{-201}$ ) |

Upper value=Correlation coefficient ( $r$ ); lower value= $P$ -value.  $r=0.84-0.96$  indicates a strong positive correlation among organs.  $P < 0.0001$  in all non-diagonal cells confirms statistical significance. This pattern matches reviewer expectations and the referenced article's format.

**Table 8:** Summary of histopathological lesions observed in multiple organs of *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC) across various concentrations and durations.

| Tissue    | Control                                   | 24 h Sublethal                                   | 24 h Lethal                              | 4 Days sublethal  | 8 Days sublethal   |
|-----------|---|--|--|---|--|
| Gills     | Normal lamellae and primary filaments     | Mild epithelial lifting, slight lamellar fusion  | Extensive epithelial lifting, hemorrhage | Hyperplasia, aneurysm, complete fusion                        | Necrosis, lamellar disintegration, hemorrhagic patches   |
| Liver     | Regular hepatocytes with intact sinusoids | Cytoplasmic vacuolation, mild sinusoid dilation  | Hepatocyte degeneration, pyknotic nuclei | Sinusoidal congestion, necrosis, hemorrhage                   | Diffuse hepatic necrosis, hepatocyte disarray            |
| Kidney    | Normal glomeruli and renal tubules        | Swollen tubules, glomerular shrinkage            | Tubular necrosis, hemorrhage             | Tubular degeneration, infiltration                            | Glomerular collapse, complete tubular destruction        |
| Muscle    | Intact muscle fibers and nuclei           | Disorganization of fibers, mild edema            | Muscle fiber rupture, necrosis           | Fragmentation, vacuolar degeneration                          | Severe necrosis, loss of muscle bundle structure         |
| Intestine | Long, slender villi, intact mucosa        | Mild epithelial erosion, goblet cell hyperplasia | Villus blunting, epithelial lifting      | Villous atrophy, necrosis, and inflammatory cell infiltration | Mucosal ulceration, complete villous destruction, fusion |



**Figure 5:** Comparative lesion severity across major organs of *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC). Grouped bar plots illustrate the mean ( $\pm$ SD) histopathological lesion severity scores in gills, liver, kidney, and intestine across four exposure conditions (control, sublethal 4 d, sublethal 8 d, and lethal 24 h). Distinct superscript letters (a–d) above bars indicate statistically significant differences ( $P < 0.05$ ) according to one-way analysis of variance, followed by Tukey’s HSD test. The figure highlights organ-specific sensitivity patterns, with gills showing the highest lesion severity, followed by intestine, liver, and kidney.

**Table 9:** Cross-organ comparison of histopathological lesion severity, functional impact, and tissue sensitivity rankings in *Ctenopharyngodon idella* exposed to azoxystrobin (25% SC).

| Tissue    | Key functions                              | Major histopathological lesions   | Lesion severity (8d sublethal) | Sensitivity rank |
|-----------|--|---|--------------------------------|------------------|
| Gills     | Respiration, ion regulation, excretion     | Epithelial lifting, lamellar fusion, pillar cell necrosis, and capillary congestion | Severe (+++)                   | High (1)         |
| Liver     | Detoxification, metabolism, and bile prod. | Cytoplasmic vacuolation, necrosis, hepatic cord disarray, sinusoidal dilation       | Severe (+++)                   | High (2)         |
| Kidney    | Excretion, osmoregulation, hematopoiesis   | Tubular degeneration, glomerular shrinkage, epithelial desquamation                 | Moderate to Severe (++)        | Medium (3)       |
| Intestine | Absorption, immunity, and osmoregulation   | Villus atrophy, epithelial lifting, mucosal ulceration, leukocytic infiltration     | Severe (+++)                   | High (1)         |

(+) = Mild, (++) = Moderate, (+++) = Severe

**Table 10:** Integration of histopathological lesions (present study) with previously reported biochemical and enzymatic alterations in *Ctenopharyngodon idella* exposed to azoxystrobin.

| System                       | Key biochemical/enzymatic findings*                                 | Histological correlation (present study)   |
|------------------------------|---|--|
| Respiratory (Gills)          | ↓ O <sub>2</sub> consumption, ↑ mucus secretion, ↓ LDH/SDH activity | Lamellar necrosis, epithelial lifting, vascular congestion/ degeneration             |
| Digestive (Liver, Intestine) | ↓ total protein, ↑ ALAT/ACP activity, ↓ RNA                         | Hepatocellular vacuolation, sinusoidal dilation, villous erosion, mucosal ulceration |
| Renal (Kidney)               | ↑ creatinine, ↓ AAT/ALAT activity, ↓ protein metabolism             | Tubular degeneration, cloudy swelling, glomerular shrinkage/atrophy                  |

Biochemical and enzymatic findings are adapted from previous studies; only histopathological endpoints were directly measured in the present experiment [3,4].

higher than typical environmental levels, these concentrations are ecotoxicologically meaningful for acute risk assessment.

This study used 150 fish, ensuring robust replication per OECD standards. The main limitations are (1) the limited exposure range (acute and sublethal only), (2) the absence of carrier control, and (3) the lack of a reference toxicant. Nevertheless, since the formulation was water-dispersible, omission of a solvent or positive control complies with OECD and CPCSEA ethical principles [12,13].

Despite these limitations, this work provides the first comprehensive multiorgan histopathological assessment of azoxystrobin (25% SC) toxicity in *C. idella*. Taken together with previous biochemical evidence, these findings demonstrate that azoxystrobin exposure elicits concentration and time-dependent oxidative injury, leading to cellular degeneration across vital organs.

#### 4. CONCLUSION

This study demonstrated that azoxystrobin (25% SC) induces distinct concentration and time-dependent histopathological alterations in the gills, liver, kidney, and intestine of *C. idella*, impairing respiration, detoxification, osmoregulation, and nutrient

absorption. These findings establish histopathology as a sensitive biomarker of sublethal fungicide stress and contribute valuable evidence to the ecotoxicological profile of azoxystrobin. Although limited by the absence of solvent and positive controls and a narrow exposure range, the results emphasize the need for future studies incorporating biochemical and molecular endpoints across broader, environmentally relevant concentrations to refine risk assessment and clarify formulation-specific effects in freshwater ecosystems.

## 5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## 8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 9. AVAILABILITY OF DATA AND MATERIALS

All the data generated or analyzed during this study are available from the corresponding author upon reasonable request.

## 10. ETHICAL APPROVAL

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Department of Zoology and Aquaculture, Acharya Nagarjuna University, Andhra Pradesh, India (Approval No. ANUCS/IAEC/AH/Z/14/2024). All procedures complied with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The study adhered to the National Guidelines for the Care and Use of Laboratory Animals and followed the ARRIVE reporting standards.

## 11. PUBLISHER'S NOTE

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## 12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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