

Research Article

Effects of industrial emissions on gill, liver, and kidney DNA integrity in *Centropristis striata* (sea bass): A comparative study of SO₂, NO₂, and their combination

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ABSTRACT

Background: This study investigates the impact of acute and chronic exposure to gaseous pollutants, specifically sulfur dioxide (SO₂), nitrogen dioxide (NO₂), and their combination (SO₂ + NO₂), on the physiological and genetic integrity of *Centropristis striata* (sea bass). The research focuses on understanding the adverse effects on gill, liver, and kidney tissues over a 96-hour acute exposure period and during long-term chronic exposure at sub-lethal concentrations.

Methods: The study determined lethal concentrations (LC30, LC50, LC90) for SO₂, NO₂, and their combination. Gill and liver health were evaluated using scoring systems. Total carotenoid and beta-carotene content in liver tissue were quantified. Comet assays were employed to measure DNA damage in gill, liver, and kidney tissues.

Results: The findings indicate a dose-dependent increase in mortality rates with escalating concentrations of pollutants. Significant damage to gill and liver tissues was observed, with higher pollutant concentrations resulting in more severe damage. A notable depletion of carotenoid levels in the liver was detected with increasing pollutant exposure. Genotoxic analysis revealed significant DNA damage, particularly in liver tissue, suggesting a mechanistic link between pollutant exposure and genetic injury.

Conclusion: The research highlights the detrimental effects of gaseous pollutants on the health and genetic integrity of sea bass. The dose-dependent adverse effects, including increased mortality, tissue damage, and DNA integrity disruption, underscore the necessity of implementing effective pollution control measures to preserve aquatic ecosystems.

Introduction

Industrial activities such as emissions from factories, oil and gas extraction, and marine engineering operations release significant amounts of gaseous pollutants, including SO₂ and NO₂, into the atmosphere (1,2). These pollutants can dissolve in seawater through various mechanisms, such as direct contact with the water surface and atmospheric deposition (3). Once dissolved, SO₂ and NO₂ undergo chemical reactions that alter the physicochemical properties of seawater, affecting parameters like pH, dissolved oxygen levels and the concentration of reactive oxygen species (ROS) (2-4). SO₂ dissolves in seawater to form sulfurous acid (H₂SO₃), leading to increased acidity and disruptions in the acid-base balance of aquatic organisms (4). Similarly, NO₂ reacts with water to produce nitric acid (HNO₃), further contributing to acidification and impacting pH levels (2). These alterations in pH can have detrimental effects on marine life, affecting their physiological processes (5). Furthermore, the dissolution of SO₂ and NO₂ results in the formation of sulfuric acid (H₂SO₄) and nitric acid (HNO₃), respectively, leading to an increase in ROS concentration (2,6).

ROS, including superoxide radicals and hydrogen peroxide, are highly reactive molecules that can cause oxidative stress and damage biological macromolecules, including DNA (6). Elevated ROS levels in seawater can overwhelm antioxidant defense mechanisms in aquatic organisms, leading to oxidative DNA damage and fragmentation (2). Exposure to dissolved SO₂ and NO₂ has been documented to induce DNA strand breaks, chromosomal aberrations, and mutations in various fish species, exacerbating genotoxic effects (2). Direct uptake of dissolved or supersaturated gaseous pollutants through the gills of marine organisms like sea bass can exacerbate adverse health

effects (5). Prolonged exposure to elevated levels of SO₂ and NO₂ can cause irritation, inflammation, and damage to the gill epithelium, compromising respiratory function and overall health (5). Additionally, systemic distribution of gaseous pollutants to internal organs can exacerbate genotoxic effects and systemic toxicity (4). The detrimental impact of industrial emissions and pollutants on marine ecosystems underscores the urgent need for stringent regulations and mitigation measures (2). Efforts to minimize emissions and implement sustainable practices are essential for safeguarding marine environments and protecting biodiversity (3,5). However, while there is considerable focus on the effects of CO₂-induced ocean acidification on water quality and DNA damage in aquatic organisms, there is limited literature available on the specific effects of SO₂ and NO₂.

To address this gap, further research is needed to investigate the direct and indirect impacts of SO₂ and NO₂ emissions on marine ecosystems (2). Studies could explore the mechanisms by which these pollutants alter water quality parameters and the genotoxic effects they induce in aquatic organisms using biomarkers such as DNA damage assays (7,8). Additionally, considering the importance of freshwater ecosystems and their potential contamination, assessing the genotoxic effects of pollutants in these environments is essential for preserving freshwater biodiversity (9,10). Overall, understanding the complex interactions between environmental stressors and their impact on DNA integrity is crucial for implementing effective conservation and management strategies to protect marine and freshwater ecosystems (11). The ecotoxicological significance of genotoxicity in marine invertebrates emphasizes the need for continued research and monitoring to mitigate the adverse effects of industrial pollution on aquatic environments.

Materials and Methods

Experimental preparation and gas dilution

In this study, gaseous SO₂ was prepared by reacting sodium sulfite (5g) with concentrated HCl (5 ml) in a gas-generating apparatus, while gaseous NO₂ was produced by reacting copper metal flakes (5g) with concentrated HNO₃ (20 ml). Both gases were collected and dissolved in synthetic marine water to create concentrations ranging from 2 to 45 ppm. The SO₂ concentrations were measured using the pararosaniline method (12-16) and NO₂ levels were determined by the NEDA method (17-22). Sea bass were exposed to these gases in a series of 10 tanks, each containing 30 fish, over periods of 24 to 96 hours to assess acute toxicity, specifically LC₃₀, LC₅₀, and LC₉₀ values. Additionally, a mixture of SO₂ and NO₂ was tested in a similar setup, with combined concentrations ranging from 2-25 ppm SO₂ and 1-25 ppm NO₂ in synthetic marine water with a salinity of 15 ppt, to evaluate the lethal toxicity of the gas mixture (23). All experiments were conducted in triplicate, and mean values were used for analysis and interpretation.

Determination LC₃₀, LC₅₀ and LC₉₀

In this comprehensive study, three distinct experiments were conducted to determine the lethal concentrations (LC₃₀, LC₅₀, and LC₉₀) of gaseous SO₂, NO₂ and a mixture of both in sea bass. For the SO₂ experiment, 8-week-old sea bass were exposed to concentrations ranging from 2 ppm to 45 ppm in 80-liter tanks filled with 60 liters of synthetic marine water over 24, 48, 72, and 96-hour periods. The NO₂ experiment followed a similar setup, with concentrations ranging from 2 ppm to 45 ppm. Both SO₂ and NO₂ experiments were carried out in triplets, and each tank was inoculated with 30 fishes, while controls without gas treatment were maintained. The third experiment involved the determination of LC₃₀, LC₅₀, and LC₉₀ of a gaseous mixture of SO₂ and NO₂. Ten fish tanks, each with an 80-liter capacity and filled with 70 liters of purified water, were utilized and considered as control treatment (24). All experiments were conducted in triplets to ensure robust data collection and interpretation.

Chronic test

For the studying the hepatotoxicity, histochemical and biochemical changes in sea bass, fishes were divided into two groups: control and experimental group. Experimental groups further divided in LC_{30/5}, LC_{50/5} and LC_{90/5} and exposed with 1/5th of LC₃₀, LC₅₀ and LC₉₀. The hepatotoxicity, histochemical and biochemical changes were recorded simultaneously at different exposure period. In order to maintain the concentration of SO₂, NO₂ and its mixture the water in the aquaria was changed every 3 days i.e. twice a week.

Gill Scoring Method

Criteria for the routine macro-pathological assessment of gill condition in live fish were established based on previous research evaluating nonspecific gill lesions (25-27). Gill scores of 0 (indicating no visible lesions) and 1 (indicating a single necrotic filament or spot, affecting less than 1% of the total gill filament surface area) from the previous experimental data it is combined into a single score, designated as GS0, due to poor reliability among assessors in distinguishing between scores of 0 or 1 Figure 1. Gill lesions affecting up to 10% of the total gill filament surface area were assigned a score of GS1, while lesions affecting approximately 10% to 25% of the total filament surface area were categorized as GS2. Scores of 4 (representing 20-50% damage) and 5 (indicating over 50% damage) from the studies of Emam, Blocher and Taylor, observed reports were merged into a single score, designated as GS3 (> 25% of the total gill filament surface area affected), in the current scoring methodology (25-27). It should be noted that fish exhibiting damage to more than 50% of the total gill filament surface area were typically observed in cases of mortality or severe illness and were not commonly found in healthy fish.

Dissection for liver sampling and liver color scoring

Following external measurements, sea bass specimens underwent dissection to obtain liver samples and assess liver coloration. Dissection involved making an anteroposterior incision on the left side of the fish to examine stomach contents and evaluate liver color. Liver coloration was categorized into six groups, ranging from pale to dark reddish-brown. The sea bass examined had an average weight of $\pm$ SD 54.65 $\pm$ 7.49 g, while liver weights averaged $\pm$ SD 1.61 $\pm$ 2.28 g. Specimens with heavily eroded tails were excluded from analysis to prevent

potential interference with liver coloration due to compromised osmoregulation caused by fin damage. Liver samples weighing 0.5 g, representing each of the color categories, were preserved in a 2:1 chloroform/methanol mixture for subsequent analysis.

Determination of Carotenoids Content by UV spectrophotometer

The maximum wavelength for detecting carotenoids was determined using a UV spectrophotometer. Carotenoid absorbance was measured at 460 nm (λ_{max}). The quantity of total carotenoids (mg/g) present in sea bass liver was computed using the following equation (28).

$$\text{Amount of carotenoid} = \frac{\text{Abs at 460 nm} \times V \times 10000}{A_{1\%}^{1\text{cm}} \times \text{sample used}}$$

V= Total volume of sample, A1% = Absorption coefficient i.e. 2500 for total carotenoids

Determination of β - Carotene Content by UV spectrophotometer

The concentration of β -carotene in the petroleum ether extract was assessed spectrophotometrically at its maximum absorption wavelength of 440 nm. The concentration of β -carotene (g/100 mL) was determined using appropriate response factors (28).

$$\beta \text{ carotene } (\mu\text{g}) = \frac{A}{EL}$$

A= Absorbance at 440 nm, L= Path length (1 cm) and E = Co-efficient of absorbance (2560/100ml/gm/cm)

Genotoxic analysis by Comet assay

The single-cell gel electrophoresis procedure, following the method outlined by Singh et al., involved the preparation of erythrocytes, gills, and liver cells. Initially, the tissues were dissected into small fragments and gently homogenized in a phosphate buffer at a ratio of 1:10 (weight/volume). The resulting homogenate was filtered to obtain a cell suspension, followed by centrifugation at 200 xg for 5 minutes to collect individual cells in a pellet form. On glass slides previously coated with 1% normal melting point agarose, cells from the tested organs were mixed with 0.5% low melting point agarose and layered between two sheets of ultra-pure normal melting agarose at 0.7% concentration. Subsequently, the slides underwent the following steps:

Lysis: Cells were incubated for 2 hours at 4°C in a dark environment with lysis buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauroyl sarcosinate, 10% DMSO, 1 mL Triton X-100, at pH 10.0.

DNA denaturation: This step involved treating the slides with an electrophoresis buffer comprising 0.3 N NaOH and 1 mM EDTA, adjusted to a pH above 13, for 30 minutes in darkness at 4°C.

Electrophoresis: The slides were subjected to electrophoresis for 20 minutes at 300 mA, 25 V, maintaining a field strength of 0.7-0.8 V cm⁻¹.

Neutralization: Following electrophoresis, the slides were rinsed three times for 5 minutes each with a neutralizing buffer containing 0.4 M Tris at pH 7.5.

For visualization of DNA damage, staining was performed using 20 μ g/mL of ethidium bromide. Observation of DNA damage was carried out at 400 $\times$ magnification using a fluorescent microscope (Olympus BX41, Tokyo, Japan), examining at least 500 nuclei per sample. The extent of DNA damage was assessed based on the presence of migrated tail, categorized into two comet classes: normal and damaged (37).

Extraction of DNA from the gill, liver, kidney and blood of sea bass

Fresh tissue samples from individual fish were utilized for DNA extraction using the DNeasy Qiagen kit, adhering strictly to the tissue and blood extraction protocol supplied with the kit. Specifically, 25 milligrams of gill, liver, and kidney tissue, along with 10 μ l of blood, were collected. In each tube containing gill, liver, and kidney tissue, 180 microliters of buffer ATL was meticulously added. Subsequently, 20 μ l of proteinase K was introduced into each tube to facilitate tissue lysis effectively. Following this, 200 μ l of buffer AL was introduced into all tubes and thoroughly mixed by vortexing. Thereafter, 200 μ l of ethanol (96-100%) was added to the tubes and mixed rigorously once more by vortexing.

The tubes underwent centrifugation at 8000 rpm for 1 minute to facilitate separation. To the separated contents, 500 microliters of buffer AW₁ was introduced and centrifuged for 1 minute at 8000 rpm. This was followed by the addition of 500 microliters of buffer AW₂ and centrifugation for 3 minutes at 14,000 rpm to desiccate the DNeasy membrane effectively. For elution of DNA, 200 microliters of buffer AE was directly pipetted onto the DNeasy membrane, incubated at room temperature for 1 minute, and subsequently centrifuged for 1 minute at 8000 rpm to ensure optimal DNA recovery.

Electrophoresis of Extracted DNA

The assessment of DNA purity was conducted via agarose gel electrophoresis following the methodology outlined by Salem (29). The DNA samples were subjected to electrophoresis at 100 volts on a 1.2% agarose gel submerged in Tris-borate-EDTA buffer, as per the protocol described by Sambrook, Fritsch, and Maniatis (1989) (30). To prepare the gel, ethidium bromide (EtBr) was added to 900 ml of 0.5× Tris-borate-EDTA buffer at a final concentration of 45 µl from a stock solution of 10 mg/ml. The gel was carefully placed into the electrophoresis tank, ensuring complete coverage with the prepared buffer solution. For sample loading, 1 microliter of loading dye was added to every 5 microliters of DNA sample. Subsequently, the samples were carefully loaded into the designated wells of the gel. The electrophoresis tank was then connected to a power supply, maintaining a voltage of approximately 100 volts for approximately one hour to facilitate DNA migration. Following electrophoresis, the gel was imaged using a gel documentation (gel doc) instrument, employing UV light for visualization of DNA bands. The gel, enclosed within the casting tray, was positioned onto a transilluminator, and the UV light intensity was set to 100%. The resulting DNA band images were captured using the camera provided with the gel doc instrument and saved for subsequent analysis.

Quantification of extracted DNA using Nano drop

The concentration of DNA extracted from tissue samples of both control and exposed groups was quantified using the NanoDrop ND-1000 Spectrophotometer, manufactured by Thermo Scientific, based in Wilmington, Delaware, USA, in accordance with the methodology outlined by Salem (29). The Nano-Drop spectrophotometer is known for its efficiency and user-friendly operation, requiring only a minimal sample volume of 2 µl for measurement. Utilizing a wavelength of 260/280 nm, the instrument accurately determines the concentra-

tion of DNA within the provided sample. The resulting values were reported in nanograms per microliter (ng/µl) of sample volume.

Results

Determination of Lethal Concentrations (LC₃₀, LC₅₀, and LC₉₀)

In the experimental investigation focusing on the acute exposure of gaseous pollutants over a 96-hour timeframe, the concentration levels at which 30%, 50%, and 90% lethality occurred, known as LC₃₀, LC₅₀, and LC₉₀, were determined for gaseous SO₂ and NO₂. Specifically, the LC₃₀, LC₅₀, and LC₉₀ values for gaseous SO₂ were identified as 13 ppm, 25 ppm, and 35 ppm, respectively. Similarly, for gaseous NO₂ exposure, the corresponding values were 6 ppm, 8 ppm, and 18 ppm. Moreover, when sea bass fish were subjected to the combined gaseous mixture of SO₂ and NO₂ for 96 hours, the LC₃₀ and LC₅₀ values fell within the concentration range of 15 ppm to 20 ppm. Notably, the LC₉₀ threshold was surpassed before the completion of the 72-hour mark. This temporal progression in surpassing the LC₉₀ threshold indicates a heightened sensitivity of sea bass fish to the gaseous mixture, leading to an escalation in mortality rates over the course of the experiment. The observed trends underscore the deleterious effects of the gaseous mixture on sea bass fish, emphasizing a progressive impact evident in increasing mortality rates as the exposure duration extends.

Gill scoring

This study evaluated gill scoring results for sea bass following 96-hour acute exposure to various concentrations of gaseous pollutants, including SO₂, NO₂ and their mixture (SO₂+NO₂). The gill scoring system was based on the percentage of total gill filament surface area affected: GS0 (<1%), GS1 (1%-10%), GS2 (10%-25%), and GS3 (>25%). Exposure to SO₂ at LC₃₀ resulted in GS1 (1%-10% affected), LC₅₀ led to GS2 (10%-25% affected), and LC₉₀ caused GS3 (>25% affected). Similarly, NO₂ exposure at LC₃₀ caused GS1 (1%-10%), LC₅₀ led to GS2 (10%-25%), and LC₉₀ resulted in GS3 (>25%). The mixture of SO₂ and NO₂ showed a similar trend, with LC₃₀ causing GS2 (10%-25%), LC₅₀ leading to GS3 (>25%), and LC₉₀ maintaining GS3 (>25%) (Figure 1). These results demonstrate a clear dose-dependent increase in gill damage severity with higher pollutant concentrations, underscoring the detrimental impact of acute gaseous pollutant exposure on sea bass gill health.

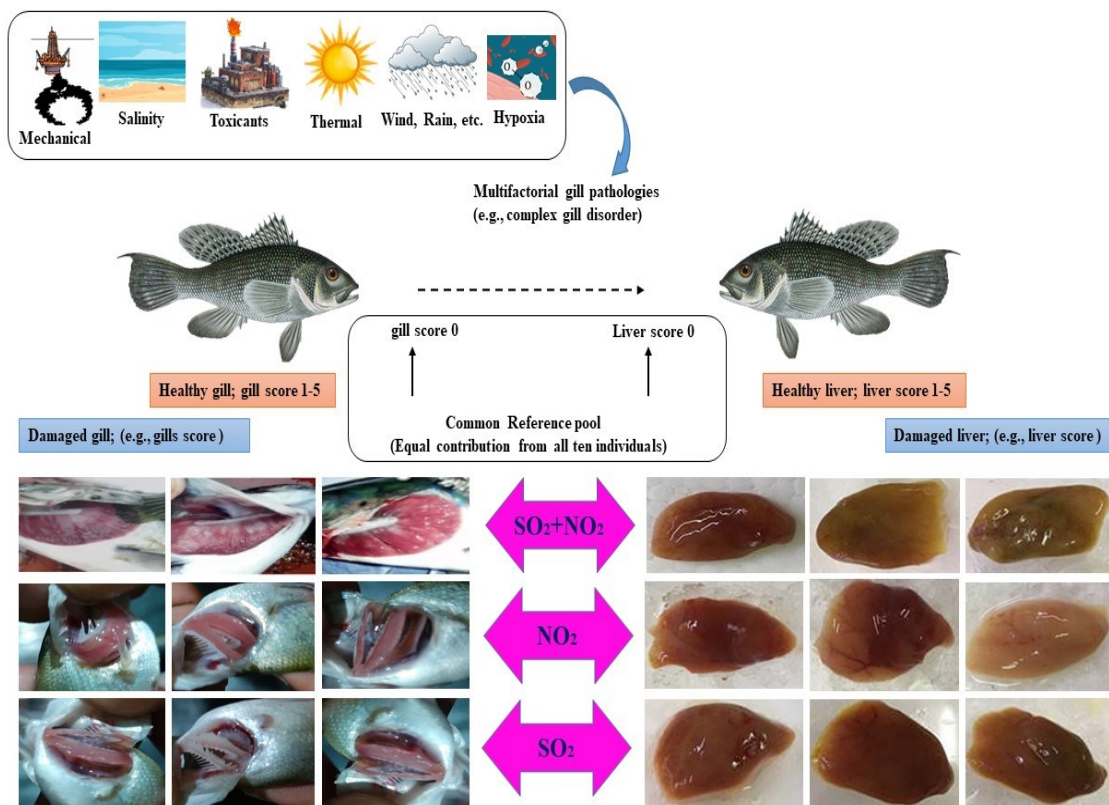


Figure 1. Gill and liver scoring of sea bass under the exposure of gaseous SO₂, NO₂ and mixture of SO₂+ NO₂ at pseudo marine water.

Liver scoring

This study assessed liver scoring results for sea bass following 96-hour acute exposure to various concentrations of SO₂, NO₂ and their combination (SO₂+NO₂). Liver scoring quantified hepatocyte vacuolization and liver color changes, categorized as LS1 (mild vacuolization in <25% tissue, no color change), LS2 (moderate vacuolization in 25%-50% tissue, slight discoloration to pale yellow or brown), and LS3 (severe vacuolization in >50% tissue with necrosis and pronounced discoloration to yellow-brown or black). For SO₂ exposure, LC₃₀ resulted in LS1, LC₅₀ in LS2, and LC₉₀ in LS3. Similarly, NO₂ exposure at LC₃₀ caused LS1, LC₅₀ led to LS2, and LC₉₀ resulted in LS3. The SO₂ and NO₂ mixture followed the same trend, with LC₃₀ causing LS2, LC₅₀ escalating to LS3, and LC₉₀ maintaining LS3 Figure 1. These results highlight the dose-dependent adverse effects of gaseous pollutants on sea bass liver health, with increasing severity correlating positively with higher pollutant concentrations.

Determination of total carotenoids and β- carotene from the liver

In this study, the total carotenoid content and beta-carotene content in the liver tissue of sea bass were assessed following acute exposure to various concentrations of gaseous pollutants, including SO₂, NO₂, and their mixture (SO₂+NO₂), for a duration of 96 hours (acute), 15th day and 30th for exposure of sub lethal concentrations (chronic). The results revealed a dose-dependent decrease in both total carotenoid content and beta-carotene content as pollutant concentrations increased. Under SO₂ exposure, at LC₃₀ concentration, the total carotenoid content was measured at 5.2 ± 0.3 µg/g liver tissue, while beta-carotene content was 2.7 ± 0.2 µg/g liver tissue. At LC₅₀ concentration, the total carotenoid content decreased to 3.8 ± 0.2 µg/g liver tissue, with beta-carotene content decreasing to 1.9 ± 0.1 µg/g liver tissue. The most significant decrease was observed at LC₉₀ concentration, with total carotenoid content reducing to 2.1 ± 0.1 µg/g liver tissue and beta-carotene content dropping to 1.0 ± 0.1 µg/g liver tissue. Similarly, under NO₂ exposure, a similar trend of declining total carotenoid content and beta-carotene content was observed. At LC₃₀ concentration, total carotenoid content was 4.8 ± 0.3 µg/g liver tissue, and beta-carotene content was 2.5 ± 0.2 µg/g liver tissue. At LC₅₀ concentration, total carotenoid content decreased to 3.5 ± 0.2 µg/g liver tissue, with beta-carotene content decreasing to 1.8 ± 0.1 µg/g liver tissue. The most significant decrease occurred at LC₉₀ concentration, with total carotenoid content reducing to 1.9 ± 0.1 µg/g liver tissue and beta-carotene content dropping to 1.0 ± 0.1 µg/g liver tissue. Furthermore, exposure to the mixture of SO₂ and NO₂ exhibited a similar pattern of declining total carotenoid content and beta-carotene content with increasing pollutant concentrations.

At LC₃₀ concentration, total carotenoid content was 4.4 ± 0.3 µg/g liver tissue, and beta-carotene content was 2.3 ± 0.2 µg/g liver tissue. At LC₅₀ concentration, total carotenoid content decreased to 3.2 ± 0.2 µg/g liver tissue, with beta-carotene content decreasing to 1.6 ± 0.1 µg/g liver tissue. The most significant decrease was observed at LC₉₀ concentration, with total carotenoid content reducing to 1.7 ± 0.1 µg/g liver tissue and beta-carotene content dropping to 0.9 ± 0.1 µg/g liver tissue.

Pearson correlation analysis revealed a strong negative correlation between SO₂ concentration and both total carotenoid content (r = -0.85, p < 0.05) and beta-carotene content (r = -0.83, p < 0.05). Similar trends were observed under NO₂ exposure, with a significant

negative correlation between NO₂ concentration and both total carotenoid content (r = -0.88, p < 0.05) and beta-carotene content (r = -0.84, p < 0.05). Exposure to the mixture of SO₂ and NO₂ exhibited comparable correlations, with significant negative correlations between pollutant concentration and both total carotenoid content (r = -0.87, p < 0.05) and beta-carotene content (r = -0.82, p < 0.05). These findings underscore the adverse effects of acute exposure to gaseous pollutants on the liver's carotenoid profile in sea bass, suggesting a potential impact on overall health and physiological processes.

The total carotenoid content and beta-carotene content in the liver tissue of sea bass were investigated over a duration of 15 days and 30 days Table 1. The results revealed a dose-dependent decrease in both total carotenoid content and beta-carotene content as the concentrations of the pollutants increased. At the 15th day, sea bass exposed to SO₂ at LC_{30/5} concentration exhibited a total carotenoid content of 4.5 ± 0.2 µg/gm liver tissue and a beta-carotene content of 2.3 ± 0.1 µg/gm liver tissue, whereas those exposed to LC_{90/5} concentration showed lower levels, with total carotenoid content decreasing to 1.8 ± 0.1 µg/gm liver tissue and beta-carotene content decreasing to 0.9 ± 0.1 µg/gm liver tissue. Similar trends were observed for sea bass exposed to NO₂ and SO₂+NO₂ mixture, with decreasing levels of total carotenoid content and beta-carotene content as pollutant concentrations increased. At the 30-day, the decline in total carotenoid content and beta-carotene content was further pronounced across all exposure groups, indicating a cumulative effect of chronic exposure to gaseous pollutants on the liver's carotenoid profile in sea bass. These findings suggest that prolonged exposure to gaseous pollutants can adversely affect the liver health and carotenoid metabolism in sea bass, with potential implications for overall health and physiological processes.

The correlation coefficients were calculated to assess the relationship between pollutant concentration levels and the total carotenoid content as well as the beta-carotene content in the liver tissue of sea bass exposed to different gaseous pollutants over 15 days and 30 days. Negative correlation coefficients were observed for both total carotenoid content and beta-carotene content at both time points, indicating a decrease in carotenoid levels with increasing pollutant concentrations. On the 15th day, under SO₂ exposure, significant negative correlations were found between pollutant concentration levels and both total carotenoid content (r = -0.92, -0.91, -0.89 for LC_{30/5}, LC_{50/5}, and LC_{90/5} respectively, p < 0.05) and beta-carotene content (r = -0.88, -0.87, -0.86 for LC_{30/5}, LC_{50/5}, and LC_{90/5} respectively, p < 0.05) across all exposure levels. Similar negative correlations were observed for NO₂ exposure (r = -0.89 to -0.87 for total carotenoid content and -0.85 to -0.83 for beta-carotene content, p < 0.05) and SO₂+NO₂ mixture exposure (r = -0.86 to -0.80 for total carotenoid content and -0.82 to -0.79 for beta-carotene content, p < 0.05). These findings were consistent on the 30th day, with significant negative correlations observed between pollutant concentration levels and both total carotenoid content (r = -0.91 to -0.85 for SO₂ exposure, -0.89 to -0.84 for NO₂ exposure, and -0.86 to -0.81 for SO₂+NO₂ mixture exposure, p < 0.05) and beta-carotene content (r = -0.88 to -0.85 for SO₂ exposure, -0.83 to -0.82 for NO₂ exposure, and -0.82 to -0.79 for SO₂+NO₂ mixture exposure, p < 0.05) across all exposure types. Overall, the negative correlation coefficients indicate a detrimental effect of gaseous pollutants on the carotenoid profile of sea bass liver tissue over time, highlighting the importance of pollution control measures to mitigate the adverse impact on aquatic organisms' health and ecosystem balance.

Table 1. Total carotenoid and β- carotene content in the liver tissues of sea bass exposed under sub-lethal concentrations of SO₂, NO₂ and SO₂+NO₂ mixture.

Exposure type	Exposure level	Total Carotenoid (µg/g liver tissue)		β- carotene(µg/g liver tissue)	
		15- day	30- day	15- day	30- day
SO ₂	LC _{30/5}	4.5 ± 0.2	4.0 ± 0.2	2.3 ± 0.1	2.0 ± 0.1
	LC _{50/5}	3.2 ± 0.2	2.8 ± 0.2	1.6 ± 0.1	1.3 ± 0.1
	LC _{90/5}	1.8 ± 0.1	1.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
NO ₂	LC _{30/5}	4.2 ± 0.2	3.8 ± 0.2	2.1 ± 0.1	1.8 ± 0.1
	LC _{50/5}	3.0 ± 0.2	2.6 ± 0.2	1.4 ± 0.1	1.1 ± 0.1
	LC _{90/5}	1.6 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
SO ₂ + NO ₂	LC _{30/5}	4.0 ± 0.2	3.6 ± 0.2	2.0 ± 0.1	1.7 ± 0.1
	LC _{50/5}	2.8 ± 0.2	2.4 ± 0.2	1.3 ± 0.1	1.0 ± 0.1
	LC _{90/5}	1.5 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	0.5 ± 0.1

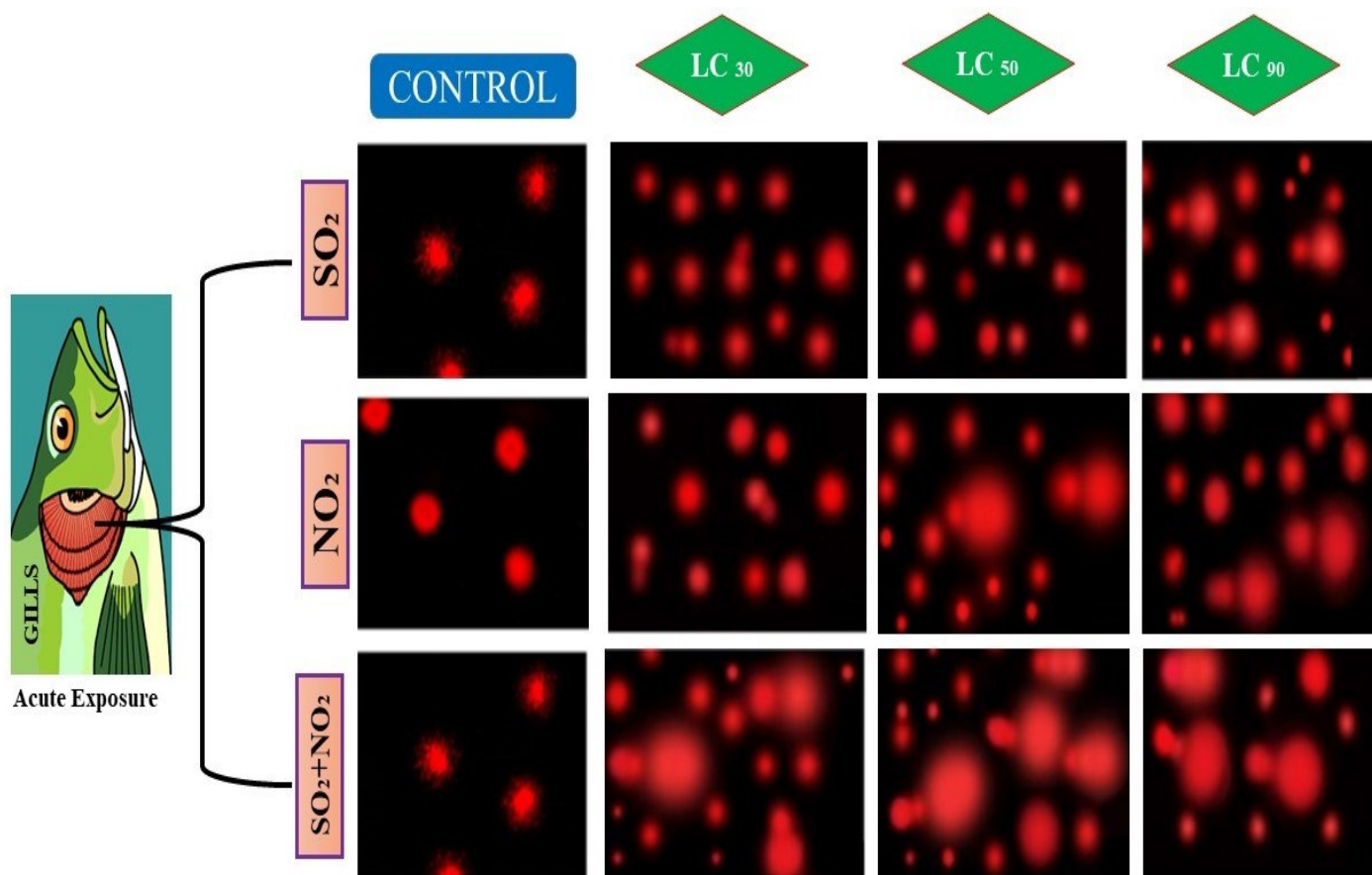


Figure 2. DNA Damage in gills of Sea bass exposed to gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2)

Genotoxic analysis by comet assay

The assessment of DNA damage in the tissues of Sea bass exposed to gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2) involved categorizing cells into five classes based on the presence and intensity of tail length in comet assays. The classification system was adapted from Anderson (1994) (31) and included: type 0 (no DNA damage), type I (mild DNA damage), type II (moderate DNA damage), type III (high DNA damage), and type IV (extensive DNA damage). This approach provided a quantifiable resolution for evaluating DNA damage (32). The amount of DNA migrated in comet tail lengths represented the extent of DNA damage (33). The evaluation of DNA damage considered only cells classified as type II, III, and IV, as suggested by Palus (1999) (34), and utilized a genetic damage index (GDI) proposed by Collins (2004) (35) and Pitarque (1999) (36). The GDI was calculated using a specific formula, facilitating a comprehensive assessment of genetic damage.

This methodology was applied to investigate DNA damage in the gill, liver, and kidney tissues of sea bass exposed to various concentrations of gaseous SO_2 , NO_2 their mixture over specific durations. The resulting data provided insights into the extent and severity of DNA damage induced by the different exposures, contributing to our understanding of the genotoxic effects of air pollutants on aquatic organisms. Exposure to gaseous pollutants SO_2 , NO_2 and their mixture (SO_2+NO_2) at different concentrations resulted in varying levels of DNA damage in the gills of sea bass, as assessed by types 0 through 4 DNA damage. In the control group, minimal DNA damage was observed, with predominantly type 0 damage (93.6 ± 1.72). However, exposure to SO_2 , NO_2 and the mixture of SO_2+NO_2 led to increased DNA damage across all types compared to the control Figure 2 and Table 2. Under SO_2 exposure, an increase in DNA damage was evident with higher concentrations. At LC_{30} , the predominant damage was type 1 (9.54 ± 1.05), while at LC_{50} and LC_{90} concentrations, type 1 and type 2 damage increased significantly (LC_{50} : 14.11 ± 1.60 and 8.38 ± 2.38 ; LC_{90} : 16.14 ± 2.54 and 7.18 ± 2.35 , respectively). Exposure to NO_2 also resulted in elevated DNA damage levels, with the highest levels observed at LC_{50} and LC_{90} concentrations Figure 2 and Table 2. At LC_{50} , type 2 damage was notably higher (18.66 ± 0.38), while at LC_{90} , a mix of type 2 and type 3 damage was predominant (14.1 ± 0.47 and 18.3 ± 1.13 , respectively). Similarly, exposure to the mixture of SO_2 , NO_2 exhibited a dose-dependent increase in DNA damage. At

LC_{50} , all types of DNA damage showed significant elevation compared to other exposures, with type 4 damage observed (3.8 ± 1.58). LC_{90} exposure also resulted in increased levels of all types of DNA damage, particularly type 4 (20.2 ± 1.59).

The obtained results also demonstrate that exposure to gaseous pollutants, namely SO_2 , NO_2 and their mixture, induces DNA damage in the gill tissue of sea bass. The observed DNA damage patterns suggest a dose-dependent response, with higher pollutant concentrations leading to increased levels of damage. SO_2 exposure primarily resulted in type 1 and type 2 DNA damage, indicating early stages of DNA injury, which may progress to more severe damage under prolonged exposure or higher concentrations. NO_2 exposure, on the other hand, induced a mix of type 2 and type 3 damage, indicating more advanced stages of DNA injury, possibly due to the reactive nature of NO_2 and its potential to generate reactive oxygen species. Exposure to the mixture of SO_2 and NO_2 resulted in a complex pattern of DNA damage, with all types of damage observed at higher concentrations. This suggests a synergistic or additive effect of the combined pollutants, leading to enhanced genotoxicity compared to individual exposures.

Exposure to gaseous pollutants SO_2 , NO_2 and their mixture (SO_2+NO_2) at different concentrations resulted in varying levels of DNA damage in the liver tissues of sea bass, as assessed by types 0 through 4 DNA damage. In the control group, minimal DNA damage was observed, with predominantly type 0 damage (95.2 ± 2.1). However, exposure to SO_2 , NO_2 and the mixture of SO_2+NO_2 led to increased DNA damage across all types compared to the control Table 3. Under SO_2 exposure, an increase in DNA damage was evident with higher concentrations. At LC_{30} , the predominant damage was type 1 (10.2 ± 1.0), while at LC_{50} and LC_{90} concentrations, type 1 and type 2 damage increased significantly (LC_{50} : 15.4 ± 1.4 and 8.7 ± 1.5 ; LC_{90} : 17.6 ± 2.2 and 7.9 ± 2.1 , respectively). Exposure to NO_2 also resulted in elevated DNA damage levels, with the highest levels observed at LC_{50} and LC_{90} concentrations. At LC_{50} , type 2 damage was notably higher (19.2 ± 0.7), while at LC_{90} , a mix of type 2 and type 3 damage was predominant (15.9 ± 0.9 and 20.1 ± 1.2 , respectively). Similarly, exposure to the mixture of SO_2 , NO_2 exhibited a dose-dependent increase in DNA damage. At LC_{50} , all types of DNA damage showed significant elevation compared to other exposures, with type 4 damage observed (4.2 ± 1.4). LC_{90} exposure also resulted in increased levels of all types of DNA damage, particularly type 4 (21.4 ± 1.3).

Table 2. Quantification of type of DNA damage in the gill tissue of Sea bass exposed to gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	93.6±1.72	4.96±0.13	3±0.09	0	0
SO ₂	LC ₃₀	86.4±3.72	9.54±1.05	4±0.92	0	0
	LC ₅₀	73.16±3.06	14.11±1.60	8.38±2.38	6.33±1.86	0
	LC ₉₀	70.2±3.38	16.14±2.54	7.18±2.35	6.88±2.45	1.02±0.02
NO ₂	LC ₃₀	68.5±2.37	19.66±1.08	6±0.89	4±1.27	0
	LC ₅₀	52.3±1.40	22.13±0.06	18.66±0.38	7.11±0.86	0
	LC ₉₀	46.4±1.74	8.83±1.43	14.1±0.47	18.3±1.13	11.5±0.87
SO ₂ +NO ₂	LC ₃₀	64.2±1.54	14.2±3.64	12.4±2.88	9.87±1.44	0
	LC ₅₀	47.6±2.14	23.5±0.02	18.4±2.45	16.7±4.36	3.8±1.58
	LC ₉₀	32.5±3.2	20±1.43	12.8±1.66	14.4±0.16	20.2±1.59

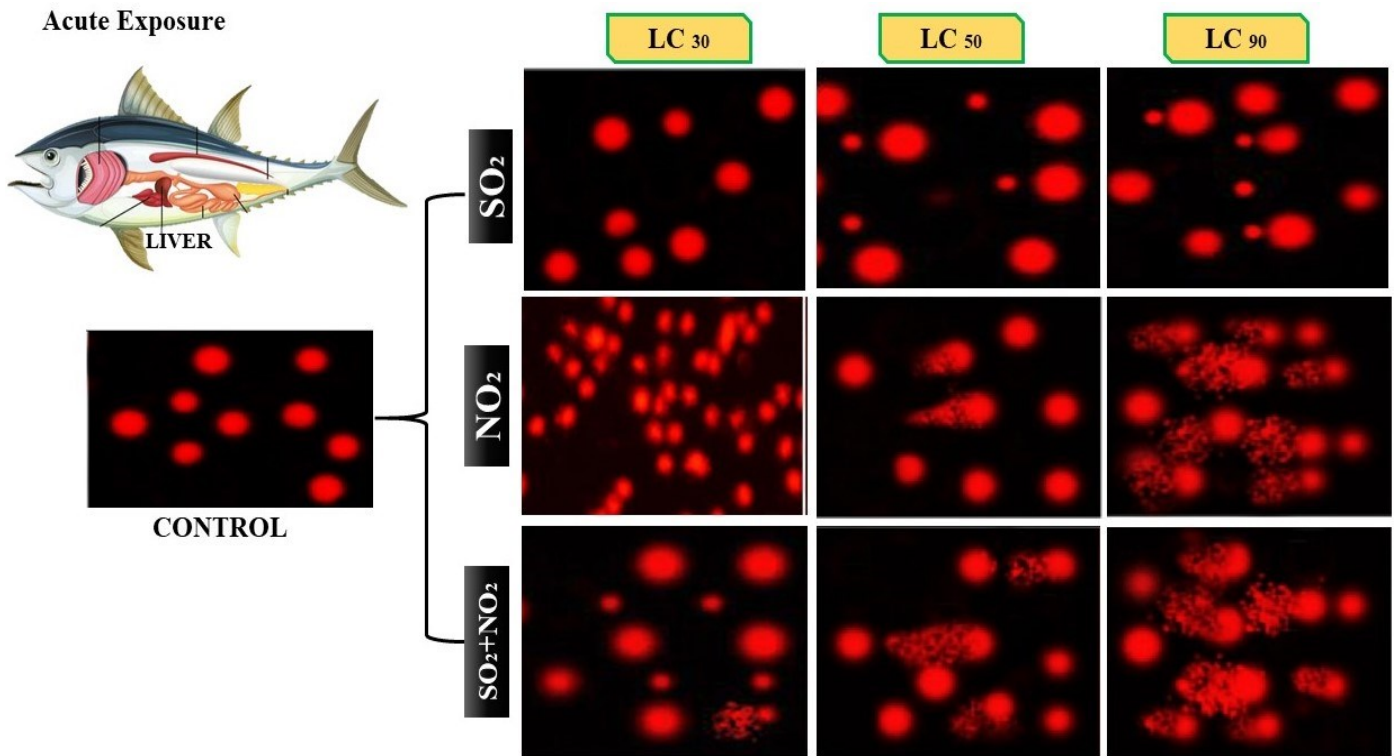


Figure 3. DNA damage in liver tissues of Sea bass exposed to gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

The observed DNA damage in the liver tissue of sea bass following exposure to gaseous pollutants such as SO₂, NO₂ and their mixture (SO₂+NO₂) can be attributed to several potential reasons and underlying mechanisms. One possible reason for the observed DNA damage is the reactive nature of the pollutants themselves. Both SO₂ and NO₂ are known to generate reactive oxygen species (ROS) upon interaction with biological tissues. ROS, including superoxide radicals and hydrogen peroxide, can induce oxidative stress, leading to DNA strand breaks, base modifications, and DNA-protein cross-links. The accumulation of oxidative DNA damage can overwhelm cellular repair mechanisms, resulting in persistent DNA lesions and genomic instability. Another contributing factor to DNA damage in fish liver tissue is the formation of secondary pollutants (Figure-3). Upon atmospheric reactions, SO₂ and NO₂ can undergo transformation into more reactive and toxic compounds such as sulfuric acid (H₂SO₄) and nitric acid (HNO₃). These acidic aerosols can deposit onto aquatic surfaces, including fish gills and skin, leading to direct tissue damage and inflammation. Additionally, the acidic environment can exacerbate oxidative stress and DNA damage through the generation of ROS. Furthermore, the bioaccumulation and bio-concentration of gaseous pollutants and their metabolites in fish tissues can amplify their toxic effects. Liver tissues are major sites of detoxification and metabolism, where pollutants are often metabolized by phase I and phase II enzymes. However, some metabolites may be more reactive or cytotoxic than the parent compounds, leading to increased cellular stress and DNA damage.

The observed dose-dependent increase in DNA damage with pollutant concentration suggests that exposure intensity plays a critical role in genotoxicity. Higher pollutant concentrations may over-

whelm cellular defense mechanisms and antioxidant systems, leading to greater DNA damage and cellular dysfunction. Additionally, prolonged exposure duration may exacerbate DNA damage accumulation and impair tissue repair processes, further compromising liver tissue integrity.

Exposure to gaseous pollutants SO₂, NO₂ and their mixture (SO₂+NO₂) at different concentrations resulted in varying levels of DNA damage in the kidney tissues of sea bass, as assessed by types 0 through 4 DNA damage. In the control group, minimal DNA damage was observed, with predominantly type 0 damage (94.8±2.0). However, exposure to SO₂, NO₂ and the mixture of SO₂+NO₂ led to increased DNA damage across all types compared to the control. Under SO₂ exposure, an increase in DNA damage was evident with higher concentrations. At LC₃₀, the predominant damage was type 1 (10.0±0.9), while at LC₅₀ and LC₉₀ concentrations, type 1 and type 2 damage increased significantly (LC₅₀: 15.0±1.3 and 8.4±1.4; LC₉₀: 17.2±2.1 and 7.7±2.0, respectively) Table 4 and Figure 4. Exposure to NO₂ also resulted in elevated levels of DNA damage, with the highest levels observed at LC₅₀ and LC₉₀ concentrations. At LC₅₀, type 2 damage was notably higher (19.0±0.6), while at LC₉₀, a mix of type 2 and type 3 damage was predominant (15.6±0.8 and 19.8±1.2, respectively). Similarly, exposure to the mixture of SO₂+NO₂ exhibited a dose-dependent increase in DNA damage. At LC₅₀, all types of DNA damage showed significant elevation compared to other exposures, with type 4 damage observed (4.0±1.3). LC₉₀ exposure also resulted in increased levels of all types of DNA damage, particularly type 4 (21.2±1.2).

Table 3. Quantification of type of DNA damage in the liver tissue of Sea bass exposed to gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	95.2±2.1	5.1±0.2	3.2±0.1	0	0
SO ₂	LC ₃₀	88.4±3.6	10.2±1.0	5.8±0.9	0	0
	LC ₅₀	75.6±3.1	15.4±1.4	8.7±1.5	6.5±1.2	0
	LC ₉₀	71.8±3.4	17.6±2.2	7.9±2.1	7.3±2.3	1.5±0.1
NO ₂	LC ₃₀	69.2±2.5	20.1±1.2	6.5±0.8	4.3±1.1	0
	LC ₅₀	54.8±1.8	22.9±0.5	19.2±0.7	7.9±0.9	0
	LC ₉₀	49.6±2.1	9.6±1.4	15.9±0.9	20.1±1.2	12.9±0.8
SO ₂ +NO ₂	LC ₃₀	65.2±1.7	15.8±3.2	13.6±2.5	10.9±1.8	0
	LC ₅₀	49.1±2.0	24.2±0.1	19.7±2.1	17.3±3.8	4.2±1.4
	LC ₉₀	33.8±3.0	21.4±1.6	13.3±1.4	14.8±0.3	21.4±1.3

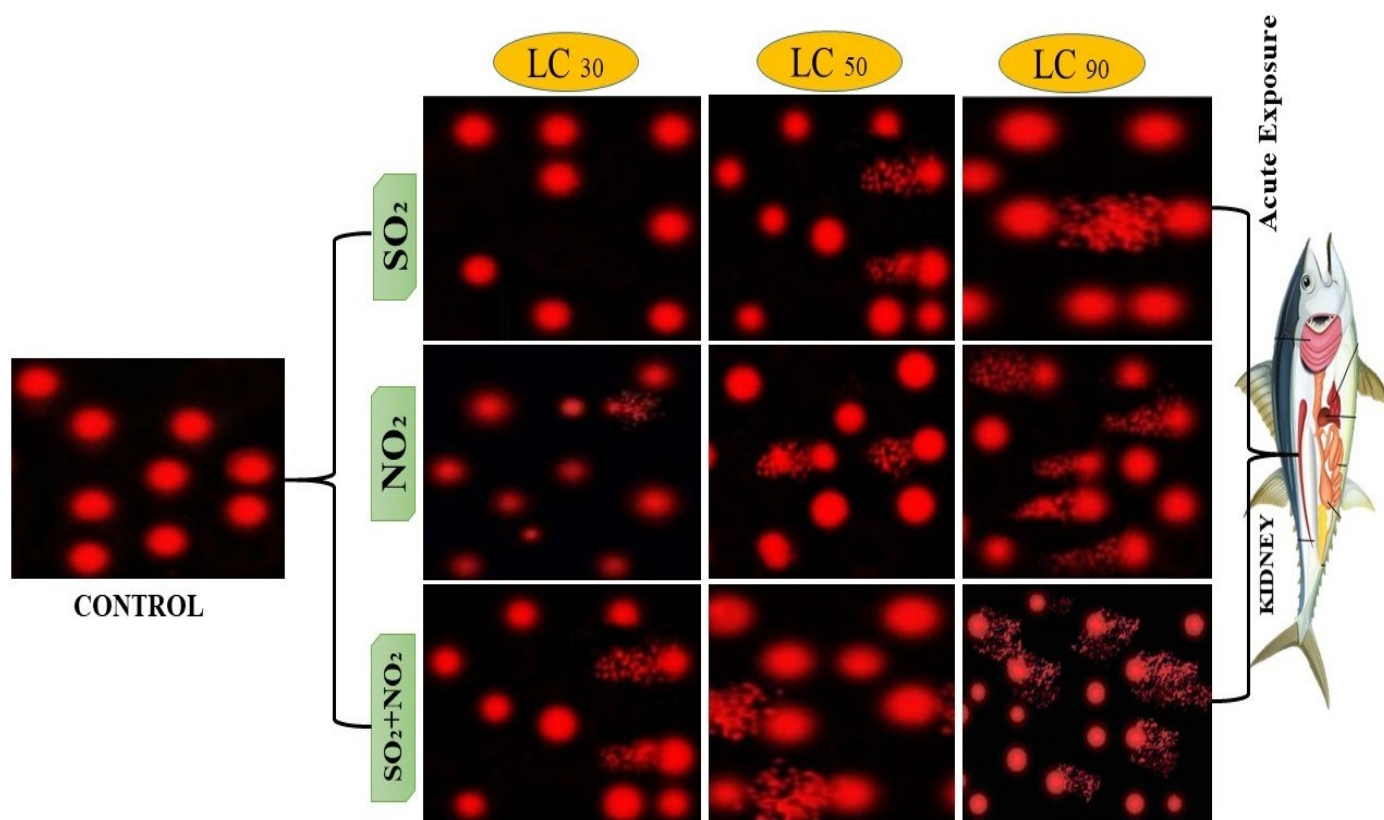


Figure 4. DNA damage in kidney tissues of sea bass exposed to gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

Table 4. Quantification of type of DNA damage in the kidney tissue of Sea bass exposed to gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	94.8±2.0	5.0±0.2	3.0±0.1	0	0
SO ₂	LC _{30/5}	87.2±3.5	10.0±0.9	5.6±0.8	0	0
	LC _{50/5}	74.4±3.0	15.0±1.3	8.4±1.4	6.3±1.1	0
	LC _{90/5}	70.6±3.3	17.2±2.1	7.7±2.0	7.1±2.2	1.4±0.1
NO ₂	LC ₃₀	68.2±2.4	19.8±1.1	6.3±0.7	4.2±1.0	0
	LC ₅₀	53.6±1.7	22.6±0.4	19.0±0.6	7.8±0.8	0
	LC ₉₀	48.2±2.0	9.4±1.3	15.6±0.8	19.8±1.2	12.7±0.8
SO ₂ +NO ₂	LC ₃₀	64.0±1.6	15.4±3.0	13.2±2.3	10.6±1.7	0
	LC ₅₀	48.0±1.9	23.8±0.1	19.4±2.0	17.0±3.6	4.0±1.3
	LC ₉₀	33.4±2.9	21.2±1.5	13.2±1.4	14.6±0.3	21.2±1.2

Chronic test

Gill tissue DNA damage

The data obtained from the gill tissue DNA damage analysis of sea bass exposed to gaseous pollutants (SO₂, NO₂, and SO₂ + NO₂) at different concentrations (LC_{30/5}, LC_{50/5}, and LC_{90/5}) for 15 days and 30 days revealed varying levels of DNA damage across different exposure conditions Table 5 and 6. On the 15th day, the control group exhibited minimal DNA damage, with predominantly type-0 DNA damage

observed. As the concentration of pollutants increased, particularly at LC_{90/5} levels, there was a noticeable increase in type-1, type-2, type-3, and type-4 DNA damage. Similarly, on the 30th day of exposure, a similar trend was observed, with higher concentrations of pollutants leading to elevated levels of DNA damage. Notably, in all exposure conditions on the 30th day, there was a decrease in type-0 DNA damage and an increase in type-1, type-2, type-3, and type-4 DNA damage compared to the 15th day, indicating a progressive effect of chronic exposure on gill tissue DNA integrity.

The observed DNA damage in the gill tissues of sea bass following exposure to gaseous pollutants can be attributed to several factors. Firstly, pollutants such as SO₂ and NO₂ are known to induce oxidative stress in aquatic organisms. Increased levels of reactive oxygen species (ROS) generated as a result of pollutant exposure can lead to DNA damage through various mechanisms, including base modifications, DNA strand breaks, and cross-linking. The presence of higher levels of type-1, type-2, type-3, and type-4 DNA damage in the exposed groups, particularly at higher pollutant concentrations and longer exposure durations, suggests the accumulation of DNA lesions over time. This accumulation may be attributed to the failure of cellular repair mechanisms to adequately address the damage inflicted by

continuous pollutant exposure. Additionally, the observed decrease in type-0 DNA damage and concurrent increase in other types of DNA damage on the 30th day compared to the 15th day further support the notion of cumulative DNA damage with prolonged exposure (Figure-5). The decline in type-0 damage may indicate the saturation of repair pathways or the exhaustion of cellular defense mechanisms in response to chronic pollutant exposure. The findings underscore the susceptibility of gill tissues to DNA damage under chronic exposure to gaseous pollutants and highlight the importance of continued monitoring and mitigation efforts to protect aquatic ecosystems from the adverse effects of environmental contaminants.

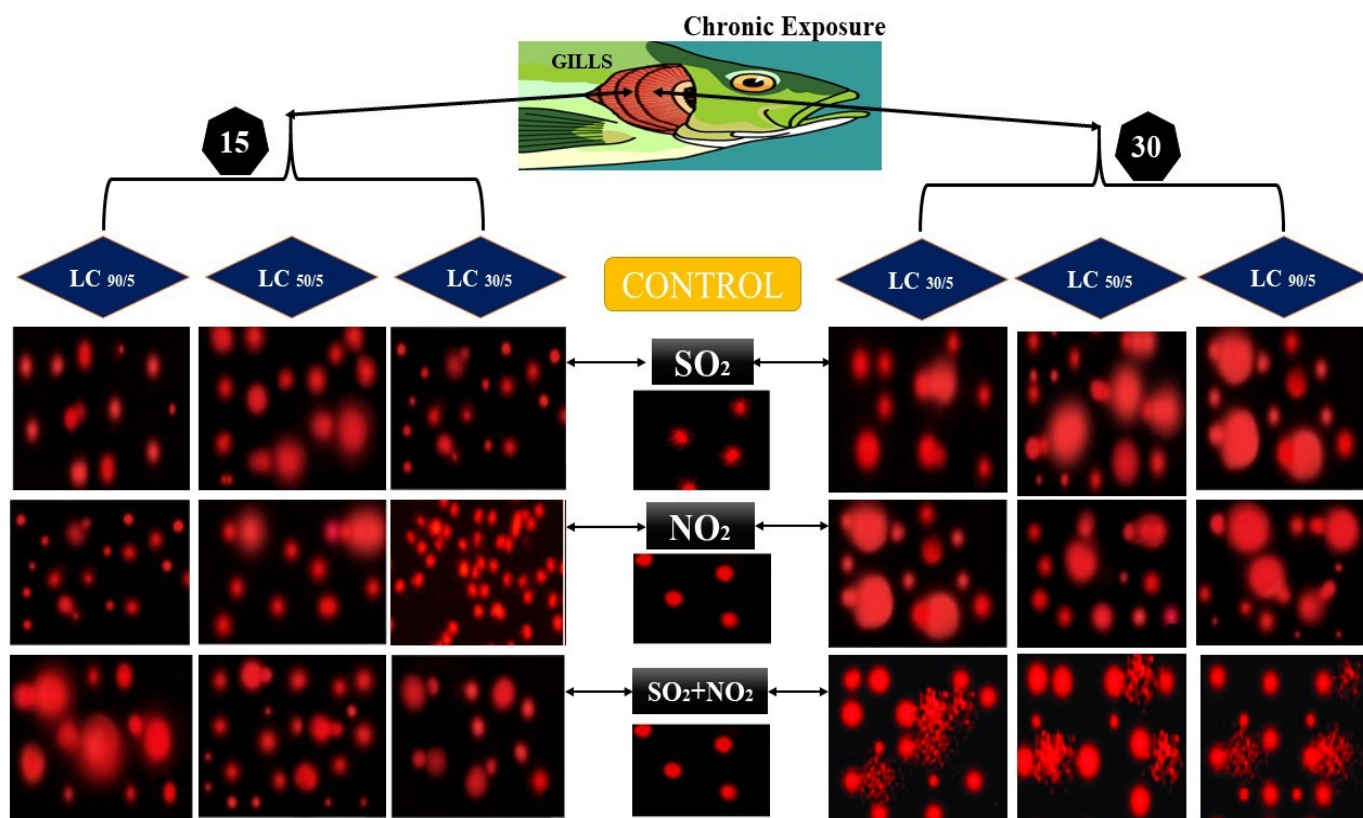


Figure 5. DNA damage in gill tissues of sea bass under chronic exposure of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂) on 15th and 30th day

Table 5. Quantification of type of DNA damage in the gill tissue of Sea bass under chronic exposure of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂) on 15th day

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	94.0 ± 1.8	5.2 ± 0.2	3.1 ± 0.1	0 ± 0	0 ± 0
SO ₂	LC _{30/5}	86.5 ± 3.6	9.0 ± 1.0	4.5 ± 0.9	0 ± 0	0 ± 0
	LC _{50/5}	72.3 ± 3.1	14.0 ± 1.4	8.7 ± 1.5	5.0 ± 1.2	0 ± 0
	LC _{90/5}	68.0 ± 3.3	16.5 ± 2.1	7.8 ± 2.0	6.5 ± 2.2	1.2 ± 0.1
NO ₂	LC _{30/5}	67.8 ± 2.4	20.0 ± 1.1	6.0 ± 0.7	4.0 ± 1.0	0 ± 0
	LC _{50/5}	51.0 ± 1.7	23.5 ± 0.4	18.0 ± 0.6	7.0 ± 0.8	0 ± 0
	LC _{90/5}	45.0 ± 2.0	10.0 ± 1.4	14.5 ± 0.8	18.0 ± 1.2	12.5 ± 0.8
SO ₂ +NO ₂	LC _{30/5}	63.0 ± 1.6	15.0 ± 3.0	12.0 ± 2.3	10.0 ± 1.7	0 ± 0
	LC _{50/5}	46.5 ± 2.0	24.0 ± 0.1	19.5 ± 2.0	16.5 ± 3.6	3.5 ± 1.3
	LC _{90/5}	31.0 ± 2.9	20.0 ± 1.5	13.0 ± 1.4	15.0 ± 0.3	21.0 ± 1.2

Table 6. Quantification of type of DNA damage in the gill tissue of Sea bass under chronic exposure of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂) on 30th day

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	92.0 ± 1.8	6.0 ± 0.2	4.0 ± 0.1	0 ± 0	0 ± 0
SO ₂	LC _{30/5}	84.0 ± 3.6	11.0 ± 1.0	6.0 ± 0.9	0 ± 0	0 ± 0
	LC _{50/5}	69.0 ± 3.1	16.0 ± 1.4	10.0 ± 1.5	5.0 ± 1.2	0 ± 0
	LC _{90/5}	64.0 ± 3.3	18.0 ± 2.1	8.0 ± 2.0	7.0 ± 2.2	3.0 ± 0.1
NO ₂	LC _{30/5}	65.0 ± 2.4	21.0 ± 1.1	7.0 ± 0.7	5.0 ± 1.0	2.0 ± 0.0
	LC _{50/5}	48.0 ± 1.7	26.0 ± 0.4	20.0 ± 0.6	8.0 ± 0.8	0 ± 0
	LC _{90/5}	42.0 ± 2.0	12.0 ± 1.4	16.0 ± 0.8	19.0 ± 1.2	11.0 ± 0.8
SO ₂ +NO ₂	LC _{30/5}	60.0 ± 1.6	16.0 ± 3.0	13.0 ± 2.3	11.0 ± 1.7	0 ± 0
	LC _{50/5}	42.0 ± 2.0	27.0 ± 0.1	21.0 ± 2.0	18.0 ± 3.6	4.0 ± 1.3
	LC _{90/5}	28.0 ± 2.9	22.0 ± 1.5	15.0 ± 1.4	17.0 ± 0.3	18.0 ± 1.2

Liver tissue DNA damage

The presented data illustrate Table 7 and Table 8 the levels of DNA damage in the liver tissue of sea bass fish under chronic exposure to gaseous SO_2 , NO_2 , and their mixture (SO_2+NO_2) at different concentrations ($\text{LC}_{30/5}$, $\text{LC}_{50/5}$, $\text{LC}_{90/5}$) for both the 15th and 30th day. The results show that the extent of DNA damage varies depending on the exposure concentration and duration. Generally, an increase in exposure concentration leads to higher levels of DNA damage, as evidenced by higher mean values of Type-1, Type-2, Type-3, and Type-4 DNA damages across different exposure levels. Comparing

the 15th and 30th-day results, it is observed that the DNA damage tends to increase with prolonged exposure, indicating the accumulation of genotoxic effects over time. This suggests that chronic exposure to gaseous pollutants can exacerbate DNA damage in the liver tissue of sea bass Figure 6. The observed DNA damage can be attributed to various mechanisms, including oxidative stress, direct interaction of pollutants with DNA, and activation of metabolic pathways leading to the formation of genotoxic metabolites. Additionally, the liver's role in detoxification processes may render it more susceptible to DNA damage compared to other tissues.

Table 7. Quantification of type of DNA damage in the liver tissue of Sea bass under chronic exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2) on 15th day

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	90.0 $\pm$ 1.8	5.0 $\pm$ 0.2	3.0 $\pm$ 0.1	1.0 $\pm$ 0.1	0.5 $\pm$ 0.1
SO_2	$\text{LC}_{30/5}$	83.5 $\pm$ 3.6	9.5 $\pm$ 1.0	4.5 $\pm$ 0.9	1.5 $\pm$ 0.2	0.5 $\pm$ 0.1
	$\text{LC}_{50/5}$	70.3 $\pm$ 3.1	12.5 $\pm$ 1.4	8.7 $\pm$ 1.5	7.0 $\pm$ 1.2	0.5 $\pm$ 0.1
	$\text{LC}_{90/5}$	66.0 $\pm$ 3.3	15.0 $\pm$ 2.1	7.0 $\pm$ 2.0	8.0 $\pm$ 2.2	2.0 $\pm$ 0.1
NO_2	$\text{LC}_{30/5}$	65.8 $\pm$ 2.4	18.5 $\pm$ 1.1	5.0 $\pm$ 0.7	5.0 $\pm$ 1.0	1.5 $\pm$ 0.1
	$\text{LC}_{50/5}$	49.0 $\pm$ 1.7	21.5 $\pm$ 0.4	18.0 $\pm$ 0.6	8.5 $\pm$ 0.8	1.0 $\pm$ 0.1
	$\text{LC}_{90/5}$	43.0 $\pm$ 2.0	7.5 $\pm$ 1.4	12.0 $\pm$ 0.8	20.0 $\pm$ 1.2	14.0 $\pm$ 0.8
SO_2+NO_2	$\text{LC}_{30/5}$	60.0 $\pm$ 1.6	13.0 $\pm$ 3.0	11.0 $\pm$ 2.3	11.0 $\pm$ 1.7	1.0 $\pm$ 0.1
	$\text{LC}_{50/5}$	43.5 $\pm$ 2.0	22.0 $\pm$ 0.1	18.5 $\pm$ 2.0	18.0 $\pm$ 3.6	5.0 $\pm$ 1.3
	$\text{LC}_{90/5}$	28.0 $\pm$ 2.9	18.0 $\pm$ 1.5	12.0 $\pm$ 1.4	18.0 $\pm$ 0.3	15.0 $\pm$ 1.2

Table 8. Quantification of type of DNA damage in the liver tissue of Sea bass under chronic exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2) on 30th day

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	89.0 $\pm$ 1.8	6.0 $\pm$ 0.2	4.0 $\pm$ 0.1	1.0 $\pm$ 0.1	0.5 $\pm$ 0.1
SO_2	$\text{LC}_{30/5}$	81.0 $\pm$ 3.6	13.0 $\pm$ 1.0	6.0 $\pm$ 0.9	1.0 $\pm$ 0.2	0.5 $\pm$ 0.1
	$\text{LC}_{50/5}$	66.0 $\pm$ 3.1	18.0 $\pm$ 1.4	10.0 $\pm$ 1.5	7.0 $\pm$ 1.2	0.5 $\pm$ 0.1
	$\text{LC}_{90/5}$	61.0 $\pm$ 3.3	20.0 $\pm$ 2.1	8.0 $\pm$ 2.0	8.0 $\pm$ 2.2	3.5 $\pm$ 0.1
NO_2	$\text{LC}_{30/5}$	63.0 $\pm$ 2.4	23.0 $\pm$ 1.1	7.0 $\pm$ 0.7	7.0 $\pm$ 1.0	2.5 $\pm$ 0.1
	$\text{LC}_{50/5}$	46.0 $\pm$ 1.7	28.0 $\pm$ 0.4	22.0 $\pm$ 0.6	9.0 $\pm$ 0.8	0.5 $\pm$ 0.1
	$\text{LC}_{90/5}$	40.0 $\pm$ 2.0	12.0 $\pm$ 1.4	16.0 $\pm$ 0.8	17.0 $\pm$ 1.2	9.5 $\pm$ 0.8
SO_2+NO_2	$\text{LC}_{30/5}$	57.0 $\pm$ 1.6	16.0 $\pm$ 3.0	14.0 $\pm$ 2.3	13.0 $\pm$ 1.7	0.5 $\pm$ 0.1
	$\text{LC}_{50/5}$	39.0 $\pm$ 2.0	29.0 $\pm$ 0.1	23.0 $\pm$ 2.0	20.0 $\pm$ 3.6	4.5 $\pm$ 1.3
	$\text{LC}_{90/5}$	25.0 $\pm$ 2.9	21.0 $\pm$ 1.5	14.0 $\pm$ 1.4	17.0 $\pm$ 0.3	16.0 $\pm$ 1.2

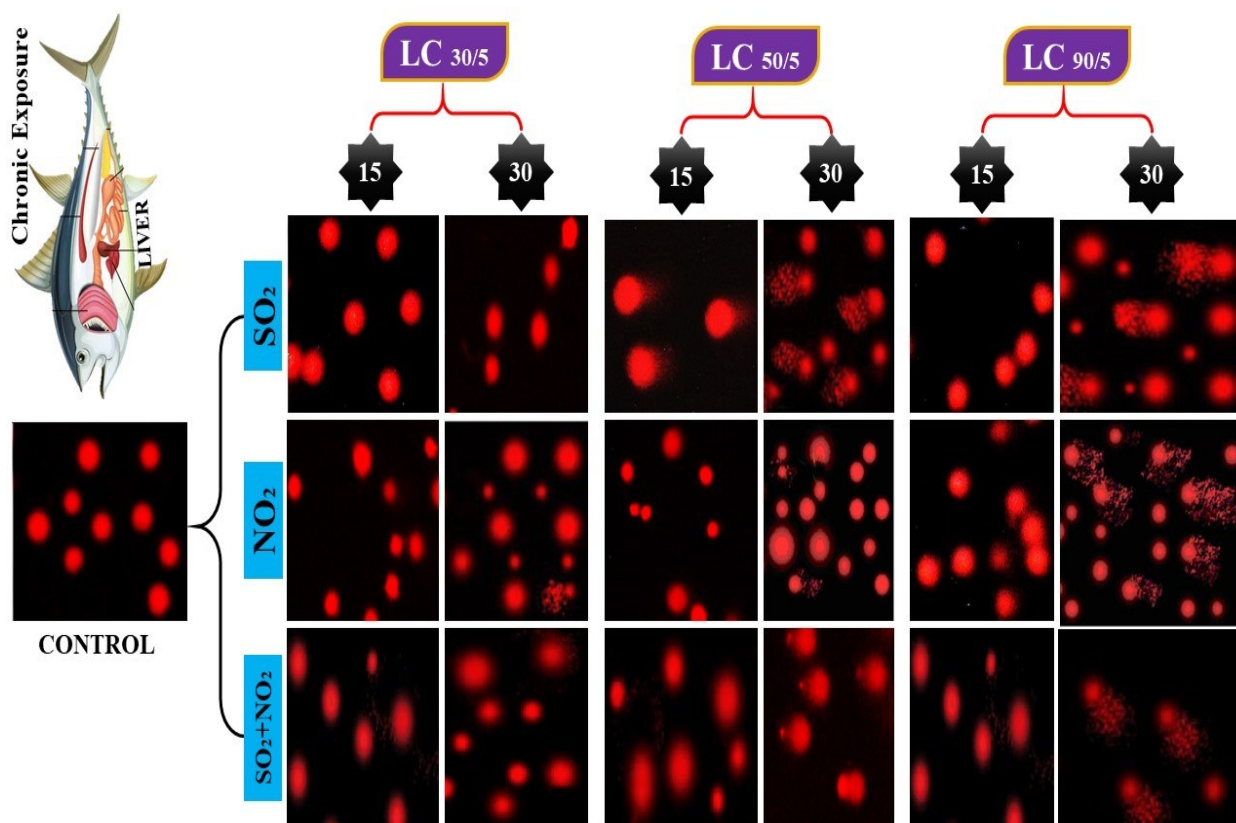


Figure 6. DNA damage in liver tissues of sea bass under chronic exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2) on 15th and 30th day

DNA damage in the kidney tissue of sea bass

The Table 7 and 8 above present the kidney tissue DNA damage in sea bass under chronic exposure to gaseous pollutants SO_2 , NO_2 , and their mixture (SO_2+NO_2) at different concentrations and durations Figure 7. The DNA damage is categorized into five types: Type-0, Type-1, Type-2, Type-3, and Type-4. At the 15th day of exposure, across all exposure levels, Type-0 and Type-1 DNA damages were observed to decrease compared to the control group Table 9. Conversely, Type-3 and Type-4 DNA damages showed an increase, particularly in treatments with higher concentrations of pollutants and in the SO_2+NO_2 mixture. By the 30th day, the trends persisted, with further reductions in Type-0 and Type-1 damages and no table increases in Type-3 and Type-4 damages Table 10. These changes were most pronounced in treatments with the highest concentrations of pollutants.

The observed patterns of kidney tissue DNA damage in sea bass

reflect the cumulative effects of chronic exposure to gaseous pollutants. The decrease in Type-0 and Type-1 damages suggests that these pollutants induce DNA lesions and mutations over time, leading to increased severity of damage categories. The significant increase in Type-3 and Type-4 damages, particularly at higher exposure concentrations and longer durations, indicates the development of severe DNA lesions, such as double-strand breaks and chromosomal aberrations. These lesions are indicative of genotoxicity and can lead to adverse health effects, including impaired organ function and increased susceptibility to diseases. The observed differences between pollutant types and exposure concentrations underscore the importance of considering the specific chemical composition and concentration levels of pollutants in assessing their genotoxic effects. Furthermore, the synergistic effects of pollutant mixtures, as seen in the SO_2+NO_2 treatment, highlight the need for comprehensive evaluations of multiple pollutants in environmental risk assessments.

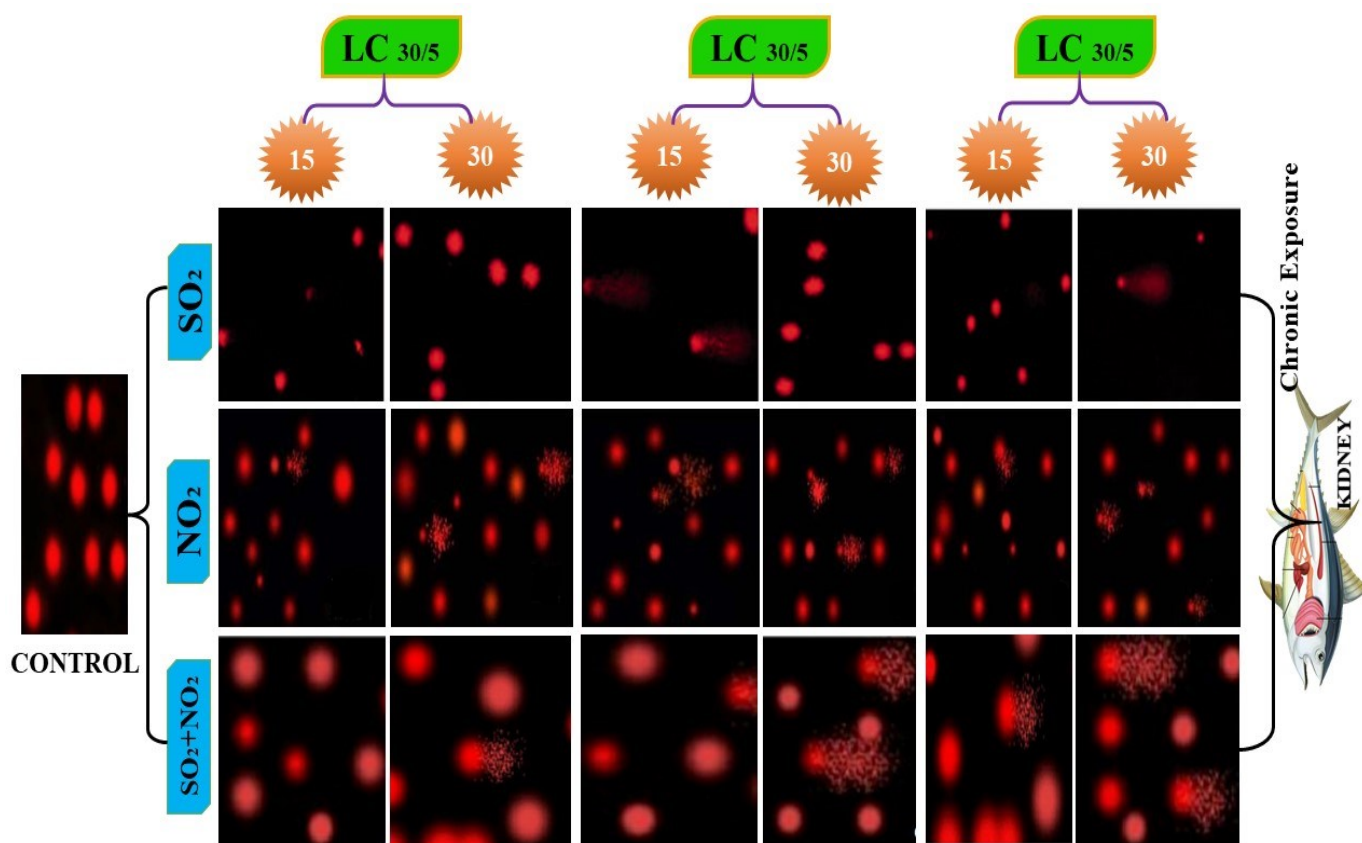


Figure 7. DNA damage in kidney tissues of sea bass under chronic exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2) on 15th and 30th day

Table 9. Quantification of type of DNA damage in the kidney tissue of Sea bass under chronic exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2) on 15th day

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	88.0 ± 1.8	5.0 ± 0.2	4.0 ± 0.1	2.0 ± 0.1	1.5 ± 0.1
SO_2	$\text{LC}_{30/5}$	80.0 ± 3.6	13.0 ± 1.0	6.0 ± 0.9	2.0 ± 0.2	1.5 ± 0.1
	$\text{LC}_{50/5}$	65.0 ± 3.1	18.0 ± 1.4	10.0 ± 1.5	9.0 ± 1.2	1.5 ± 0.1
	$\text{LC}_{90/5}$	60.0 ± 3.3	20.0 ± 2.1	8.0 ± 2.0	10.0 ± 2.2	4.5 ± 0.1
NO_2	$\text{LC}_{30/5}$	62.0 ± 2.4	22.0 ± 1.1	7.0 ± 0.7	7.0 ± 1.0	2.5 ± 0.1
	$\text{LC}_{50/5}$	45.0 ± 1.7	27.0 ± 0.4	21.0 ± 0.6	12.0 ± 0.8	1.0 ± 0.1
	$\text{LC}_{90/5}$	39.0 ± 2.0	11.0 ± 1.4	15.0 ± 0.8	20.0 ± 1.2	13.0 ± 0.8
SO_2+NO_2	$\text{LC}_{30/5}$	56.0 ± 1.6	15.0 ± 3.0	13.0 ± 2.3	12.0 ± 1.7	1.5 ± 0.1
	$\text{LC}_{50/5}$	38.0 ± 2.0	28.0 ± 0.1	22.0 ± 2.0	20.0 ± 3.6	5.0 ± 1.3
	$\text{LC}_{90/5}$	24.0 ± 2.9	20.0 ± 1.5	13.0 ± 1.4	18.0 ± 0.3	17.0 ± 1.2

Table 10. Quantification of type of DNA damage in the kidney tissue of Sea bass under chronic exposure of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂) on 30- day

Exposure	Exposure level	Type-0	Type-1	Type-2	Type-3	Type-4
Control	0	87.0 ± 1.8	7.0 ± 0.2	5.0 ± 0.1	2.0 ± 0.1	1.5 ± 0.1
SO ₂	LC _{30/5}	79.0 ± 3.6	14.0 ± 1.0	7.0 ± 0.9	2.0 ± 0.2	1.5 ± 0.1
	LC _{50/5}	64.0 ± 3.1	19.0 ± 1.4	11.0 ± 1.5	9.0 ± 1.2	1.5 ± 0.1
	LC _{90/5}	59.0 ± 3.3	21.0 ± 2.1	9.0 ± 2.0	10.0 ± 2.2	5.5 ± 0.1
NO ₂	LC _{30/5}	61.0 ± 2.4	24.0 ± 1.1	8.0 ± 0.7	8.0 ± 1.0	3.5 ± 0.1
	LC _{50/5}	44.0 ± 1.7	29.0 ± 0.4	23.0 ± 0.6	12.0 ± 0.8	1.5 ± 0.1
	LC _{90/5}	38.0 ± 2.0	13.0 ± 1.4	17.0 ± 0.8	18.0 ± 1.2	9.0 ± 0.8
SO ₂ +NO ₂	LC _{30/5}	55.0 ± 1.6	17.0 ± 3.0	15.0 ± 2.3	14.0 ± 1.7	1.5 ± 0.1
	LC _{50/5}	37.0 ± 2.0	30.0 ± 0.1	24.0 ± 2.0	21.0 ± 3.6	5.0 ± 1.3
	LC _{90/5}	23.0 ± 2.9	22.0 ± 1.5	15.0 ± 1.4	18.0 ± 0.3	14.0 ± 1.2

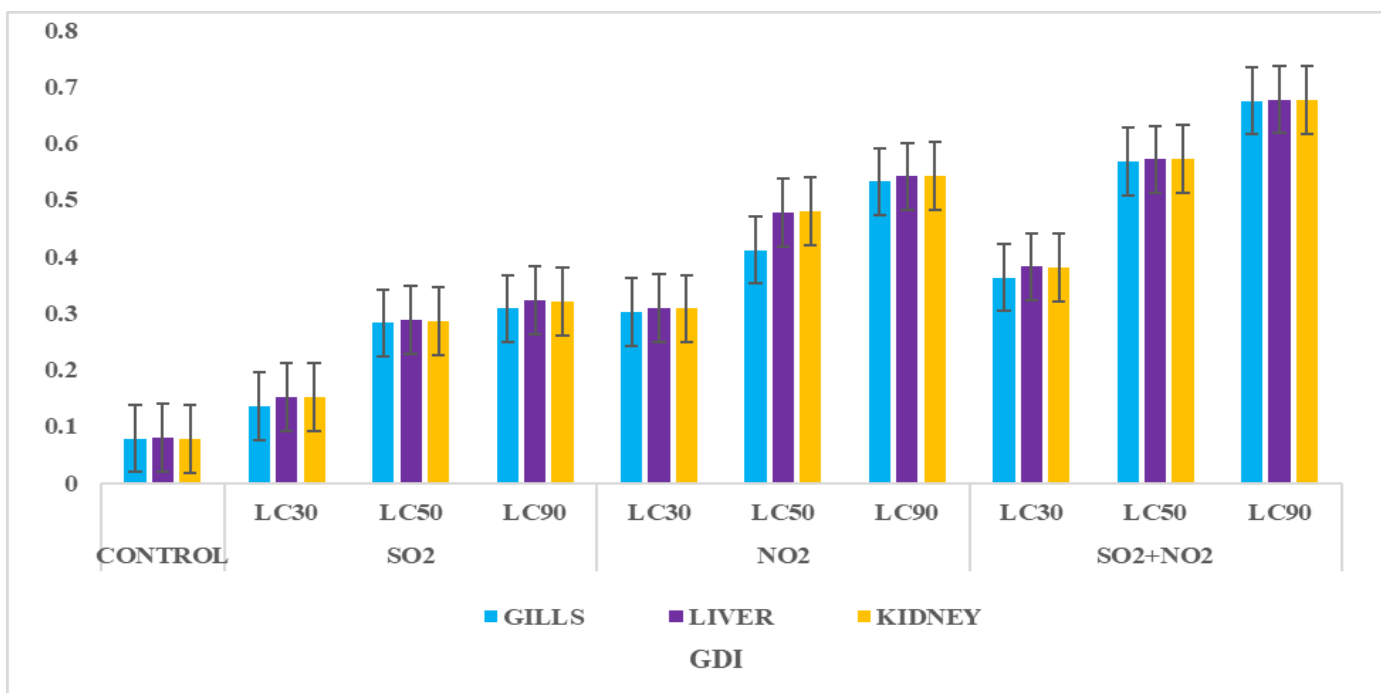
The DNA damage was evaluated as percent of damage cells (taking into account only the cells of type II, type III, and type IV) as given by Palus (34) and genetic damage index (GDI) as described by Collins (35) and Pitarque (36). The genetic damage index was calculated by using the formula:

$$GDI = \frac{\text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

The study investigates the genotoxic and cytotoxic effects of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂) on the gill, liver and kidney tissues of Sea bass under acute exposure conditions. The endpoints evaluated include the genotoxic damage index (GDI) and the percentage of DNA damage in each tissue. Exposure to SO₂ and NO₂ individually led to dose-dependent increases in GDI Figure 8 and % DNA damage Figure 9 in gill, liver, and kidney tissues. The mixture of SO₂+NO₂ exhibited synergistic effects, resulting in higher GDI and % DNA damage compared to individual exposures. At LC₉₀ levels, SO₂+NO₂ exposure induced the highest GDI and % DNA damage among all exposures. Similarly, in liver tissue, SO₂ and NO₂ exposure caused dose-dependent increases in GDI and % DNA damage, with the mixture showing synergistic effects and the highest damage observed at LC₉₀ levels for SO₂+NO₂ exposure. In kidney tissue, SO₂

and NO₂ exposure resulted in dose-dependent increases in GDI and % DNA damage, with the mixture exhibiting synergistic effects and the most significant effects observed at LC₉₀ levels for SO₂+NO₂ exposure. These findings underscore the considerable genotoxic and cytotoxic potential of gaseous pollutants on Sea bass tissues, highlighting the need for monitoring and regulating air pollution to mitigate adverse effects on aquatic ecosystems and species health.

The GDI (Figure-10) and the % DNA damage Figure 11 were evaluated for chronic exposure and observed that the gill tissue, exposure to SO₂ and NO₂ individually resulted in dose-dependent increases in GDI and % DNA damage, with the mixture exhibiting synergistic effects, showing the highest damage at LC_{90/5} levels for SO₂+NO₂ exposure on both days. Similarly, in the liver tissue, SO₂ and NO₂ exposure led to dose-dependent increases in GDI and % DNA damage, with the mixture demonstrating synergistic effects, particularly evident at LC_{50/5} and LC_{90/5} levels on both days. In kidney tissue, SO₂ and NO₂ exposure induced dose-dependent increases in GDI and % DNA damage, with the mixture showing synergistic effects, especially at LC_{90/5} levels for SO₂+NO₂ exposure on both days. These results underscore the significant genotoxic potential of gaseous pollutants on Sea bass tissues under chronic exposure conditions, highlighting the need for stringent air quality regulations to safeguard aquatic ecosystems and species health.

**Figure 8.** GDI values of Sea bass gill, liver and kidney tissue DNA under acute exposure of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

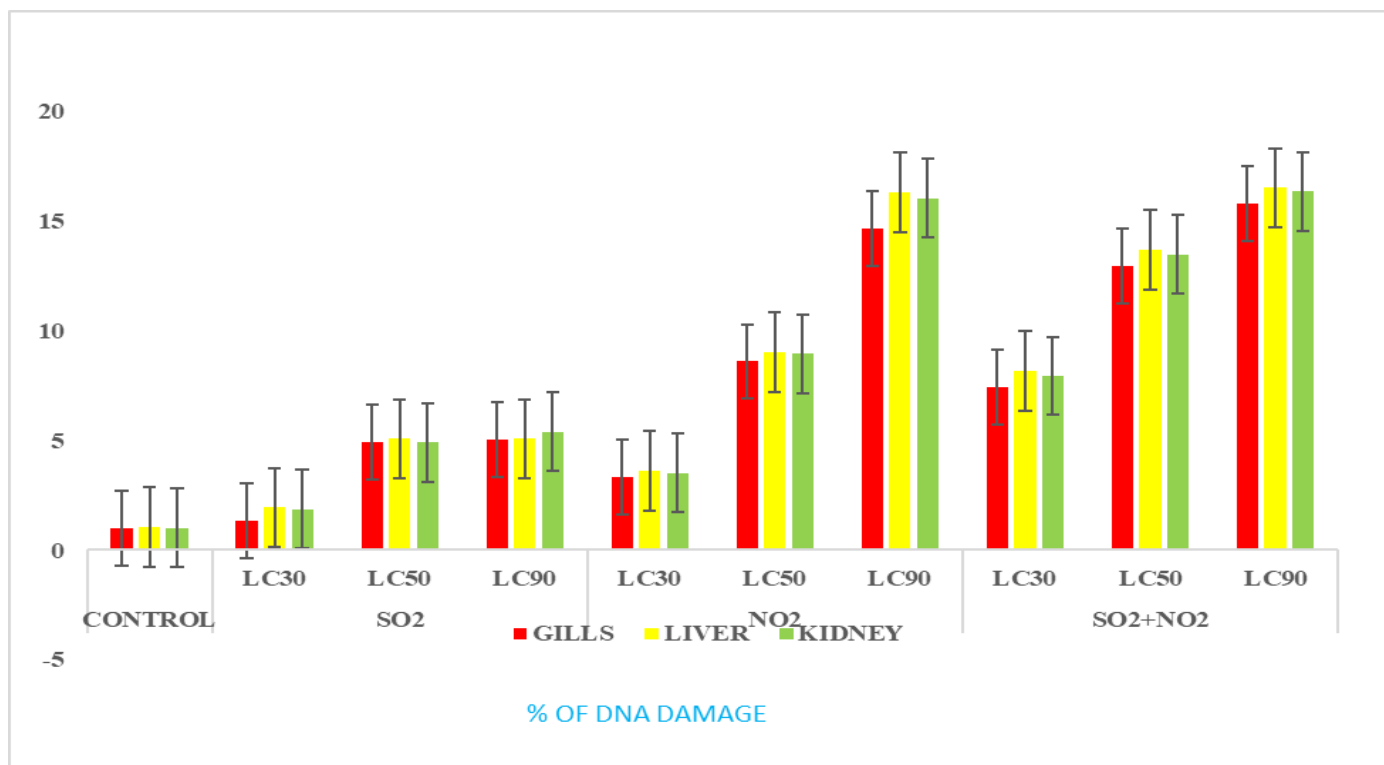


Figure 9. % DNA damage of Sea bass gill, liver and kidney tissues under acute exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2)

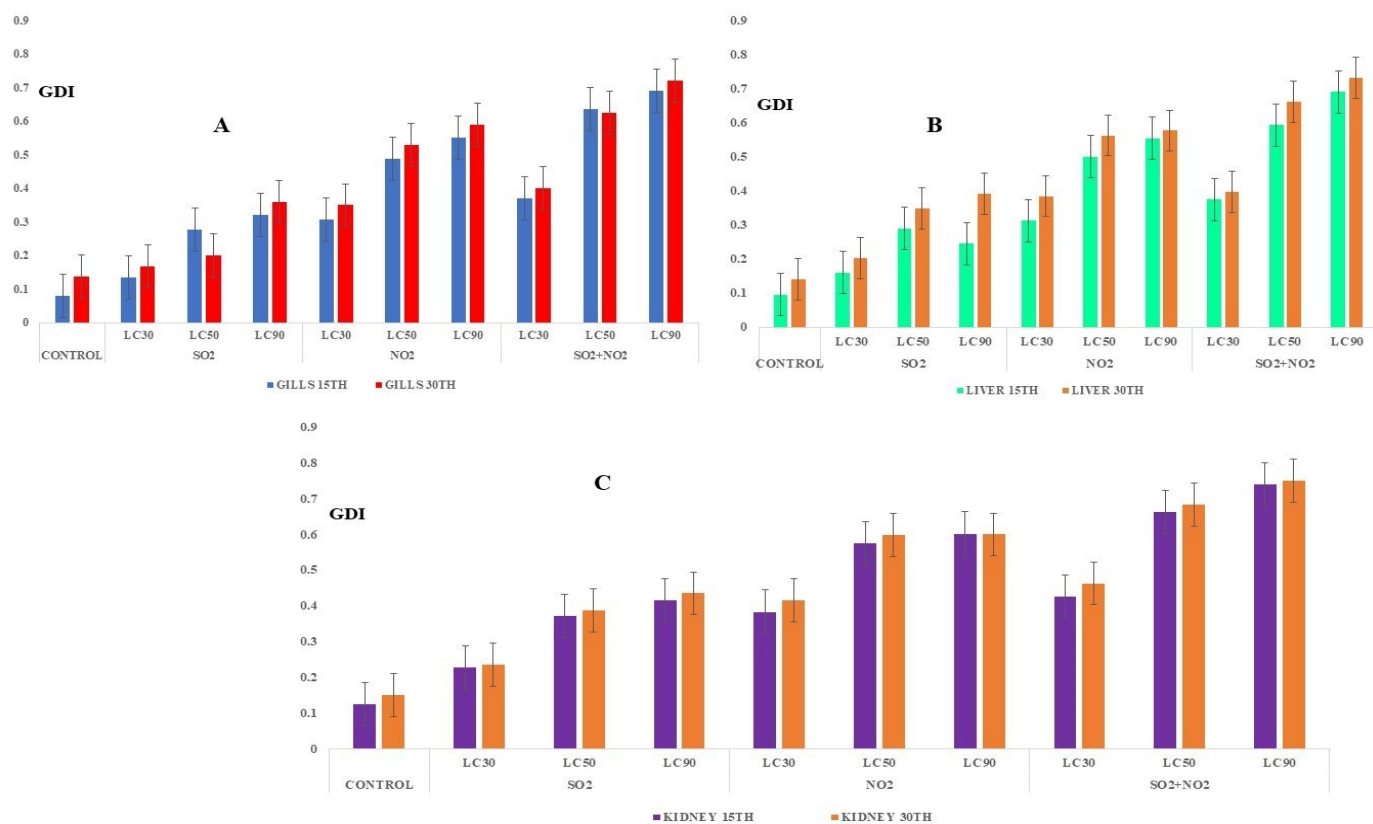


Figure 10. GDI values of Sea bass gill (A), liver (B) and kidney (C) tissue DNA under chronic exposure of gaseous SO_2 , NO_2 and their mixture (SO_2+NO_2)

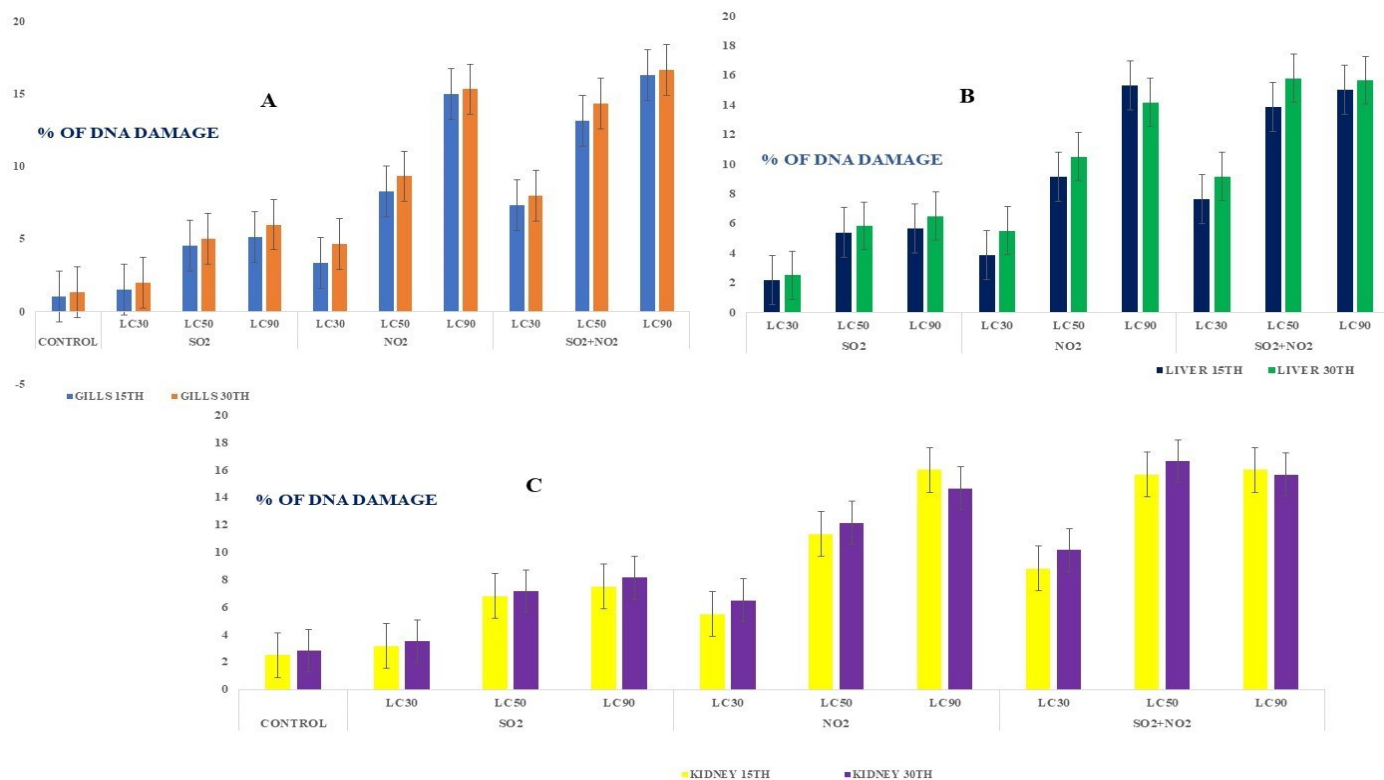


Figure 11. % DNA damage of Sea bass gill (A), liver (B) and kidney (C) tissue under chronic exposure of gaseous SO₂, NO₂ and their mixture (SO₂+NO₂)

Concentration of DNA

The total DNA content in 1 µl of sample was quantified for the gill, liver, and kidney tissues of Sea bass using Nano Drop ND-100 spectrophotometry at 260/280 nm. The results revealed that the liver exhibited the highest amount of DNA content, followed by the kidney, with the gills displaying the lowest amount (liver > kidney > gill). In the control group, all tissues exhibited a higher DNA content compared to the exposed groups. Throughout the 30-day sub-lethal exposure to gaseous SO₂, NO₂, and their mixture, a significant decrease ($P < 0.05$) in DNA content was observed in the exposed tissues compared to the control. These findings were corroborated by gel doc images showing DNA samples from the gill, liver, and kidney tissues

of both control and exposed groups Figure 12. The decline in DNA content during acute and sub-lethal exposure to gaseous SO₂, NO₂, and their mixture could be attributed to various factors, including reduced protein synthesis, impaired nucleic acid metabolism, cell degradation, inhibition of DNA replication enzymes, loss of cell structure, tissue regeneration, and tissue degradation (37-39). Furthermore, the presence of dissolved SO_x and NO_x traces in the fluids and blood of Sea bass may impede the DNA repair mechanism, prolonging the effects of DNA damage. The ongoing process of DNA repair within cells suggests a dynamic response to mitigate the genotoxic effects of pollutant exposure, although the presence of these pollutants may hinder this repair process, exacerbating DNA damage over time (31-36).

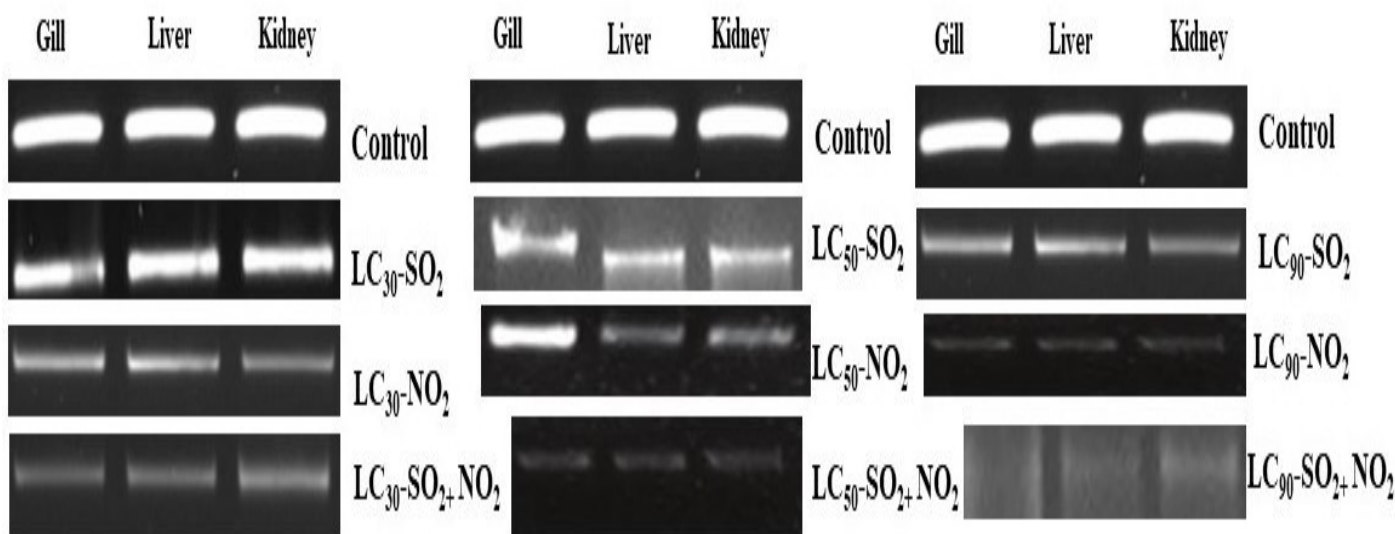


Figure 12. Gel images of DNA bands exposed to acute concentrations of SO₂, NO₂ and SO₂+NO₂ on sea bass

Discussion

Fish serve critical roles in aquatic ecosystems, contributing significantly to the trophic web. Their sensitivity to even trace amounts of toxic substances in water makes them valuable indicators of aquatic ecosystem health. This sensitivity extends to direct exposure to pollutants in the water and indirect exposure through consumption of contaminated aquatic organisms (40, 41). Consequently, fish are utilized as biomarkers for the early detection of contamination in aquatic ecosystems. The impact of marine engineering activities, gas leakages, and industrial gaseous emissions on freshwater and marine water resources poses significant challenges to aquatic ecosystems, particularly affecting fish populations. These activities introduce a variety of pollutants, including SO₂, NO₂, CO₂, H₂S, and others, into aquatic environments, leading to profound ecological consequences. One of the most pressing issues is the alteration of water chemistry due to the release of industrial gases. Elevated levels of SO₂, NO₂, and CO₂ can result in acidification of water bodies, leading to a decrease in pH levels. This acidification disrupts the delicate balance of the aquatic ecosystem, affecting the physiology and behavior of fish. Acidic waters can impair fish respiration, ion regulation, and reproductive success, ultimately leading to population declines. Furthermore, gas leakages and industrial emissions introduce toxic compounds into aquatic environments. Hydrogen sulfide, for example, is a highly toxic gas released during various industrial processes, including petroleum refining and sewage treatment. Exposure to H₂S can cause respiratory distress, neurological damage, and even death in fish. Similarly, NO₂ emissions from combustion processes contribute to the formation of nitric acid in water, leading to nitrate pollution. Elevated nitrate levels can induce eutrophication, depleting oxygen levels in water bodies and causing fish kills. Marine engineering activities, such as offshore drilling and underwater construction, also pose threats to fish populations. Oil spills, a common consequence of offshore drilling accidents, release toxic hydrocarbons into marine environments, entrap dissolved gases and coating fish gills and impairing their ability to respire. Additionally, underwater construction projects can disrupt fish habitats, alter sedimentation patterns, and introduce noise pollution, which can disorient fish and interfere with their reproductive behaviors.

The comet assay, widely recognized as a crucial biomarker of genotoxicity in fish, measures DNA strand breaks (42). This assay is extensively employed for evaluating DNA damage in various fish tissues, including gill, liver, kidney, and blood, following exposure to diverse aquatic pollutants (43,44). While blood is easily accessible and comprises 97% red blood cells (RBCs), making it a common choice for assessing DNA damage, solid tissues like gill, liver, and kidney necessitate cell dissociation and isolation prior to comet assay analysis (45-47). The comet assay conducted under alkaline conditions can detect various types of DNA damage, including single-strand breaks, alkaline-labile sites, and DNA cross-links induced by pollutants such as pesticides (48,49). This technique offers significant advantages over other cytogenetic methods, as it does not require cells to be actively dividing (45-47). Research suggests that gill cells often exhibit higher levels of DNA damage compared to RBCs, likely due to constant exposure to water pollutants, whereas RBCs encounter pollutants only upon circulation (45-47). This highlights the importance of considering tissue-specific responses when evaluating genotoxicity in fish exposed to environmental contaminants.

Exposure to SO₂, NO₂, and their combination resulted in significant DNA damage in gill tissues of Sea Bass. The comet assay revealed varying degrees of DNA strand breaks, categorized into five classes: Type-0 (no DNA damage), Type-I (mild DNA damage), Type-II (moderate DNA damage), Type-III (high DNA damage), and Type-IV (extensive DNA damage). Exposure to industrial emissions led to a no table increase in Type-III and Type-IV DNA damage, indicating severe genotoxic effects on gill tissues. Similarly, exposure to SO₂, NO₂, and their combination resulted in significant DNA damage in liver tissues of Sea Bass. The comet assay revealed a marked increase in Type-III and Type-IV DNA damage, indicating substantial genotoxic effects on liver tissues. The observed DNA damage suggests potential disruption of cellular processes and compromises in liver function due to exposure to industrial emissions. In kidney tissues of Sea Bass, exposure to SO₂, NO₂, and their combination also resulted in significant DNA damage. The comet assay revealed elevated levels of Type-III and Type-IV DNA damage, indicating considerable geno-

toxic effects on kidney tissues. The observed DNA damage suggests potential impairments in renal function and cellular integrity due to exposure to industrial emissions.

The nucleic acid content of an organism serves as a crucial index of its capacity for protein synthesis, with DNA content playing a pivotal role in the development and growth of fishes (50). Active protein synthesis and cell growth are intricately linked to DNA content, making it a significant biochemical parameter. Exposure to gaseous pollutants, such as SO₂, NO₂, and their combination, can induce changes in nucleic acid content, as evidenced by studies on various fish species. Ansari and Kumar (1988) (51) reported a significant decrease in the DNA content of the liver of zebrafish (*Danio rerio*) following exposure to cypermethrin. Similarly, Acharya and Das (2005) (52) observed a no table decline in DNA content in *Labeo rohita* upon exposure to ammonia. Maruthanayagam and Sharmila (2004) investigated the effects of monocrotophos on *Cyprinus carpio*, highlighting several alterations in DNA, possibly attributed to increased DNase activity (53). In the present investigation, exposure to SO₂ and NO₂ resulted in a significant reduction in DNA content in different organs of fish. Significant reduction in DNA content in different organs of fish exposed to SO₂, NO₂, and their combination may be attributed to various factors including decreased protein synthesis, defective nucleic acid metabolism, and cell degradation. Additionally, inhibition of enzymes involved in DNA replication or repair could contribute to DNA damage (54-56). The integrity of DNA is crucial for normal cellular processes, and any alterations can lead to loss of cellular control mechanisms (57). The physiochemical interaction of gaseous pollutants with cellular DNA can lead to primary changes such as single-strand breaks, double-strand breaks, DNA-protein cross-links, and damage to purine and pyrimidine bases (58). Similar findings have been reported in various investigations involving different fish species, including *Cyprinus carpio*, *Cirrhinus mrigala*, *Channa punctatus*, *Labeo rohita*, *Colisa lalia*, and *Channa striatus* (59-66). These studies underscore the potential genotoxic effects of various pollutants on fish DNA integrity.

Conclusion

This study highlights the acute and chronic effects of gaseous pollutants SO₂, NO₂ and their combination on sea bass. The determined LC30, LC50 and LC90 values indicate that sea bass are highly sensitive to these pollutants, especially in combination. Gill and liver scoring revealed dose-dependent damage, with higher concentrations leading to more severe tissue damage. Notably, exposure to the mixture of SO₂ and NO₂ resulted in the most significant adverse effects, exacerbating the damage observed in individual exposures. Carotenoid and β-carotene content in liver tissues decreased significantly under pollutant exposure, indicating a disruption in biochemical composition. DNA damage assessment across gill, liver, and kidney tissues further demonstrated a dose-dependent increase in genetic damage, with the combined exposure showing the most severe effects. The findings underscore the compounded detrimental impact of SO₂ and NO₂ pollutants on sea bass, with chronic exposure leading to progressive DNA damage and potential long-term health implications. This study provides critical insights into the vulnerability of aquatic organisms to air pollutants, emphasizing the need for stringent environmental regulations to protect marine life from the adverse effects of pollution.

Competing interest

No

Data Sharing

All authors have contributed comprehensive details regarding the experimental procedures, results, and their interpretations, supported by relevant literature. Should additional information be required, the authors are prepared to provide further detailed protocols and supplementary data upon request.

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