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Oxidative Stress-Induced Micronuclei in Common Carps in Physicochemically Contaminated Pond

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Abstract

It is a general practice to engage in waterbodies with anthropogenic activities, which can alter the physicochemical nature of the water and introduce various types of pollutants. Several types of pollutants are responsible for inducing oxidative stress in aquatic organisms, leading to tissue and nucleic acid damage, and ultimately resulting in significant mortality and economic losses to aquaculture. In the present study, the physicochemical parameters of the anthropogenically involved pond were analyzed for all four seasons (summer, monsoon, post-monsoon, and winter). The oxidative stress (DPPH, FRAP, and $H_2 0_2$) in two major carps (*Labeo rohita* and *Catla catla*) was studied, and the results of oxidative stress in the form of micronucleus development were analyzed to establish the mortality of carps. The lower alterations in physicochemical concentrations were reported in the monsoon season and were maximum in the winter season. The oxidative stress was also reported as maximum in winter and minimum in the monsoon season in both carp. Relative micronuclei have also been reported to be comparatively higher in *C. catla* than the *L. rohita*. The findings of the study provide substantive pieces of information not only for the effect of pollutants on fish but also give an idea of the suitability of species for aquaculture.

Keywords: Oxidative stress, DPPH, FRAP, H_2O_2, Micronuclei, Aquatic pollutants, *Labeo rohita, Catla catla*.

INTRODUCTION

The water bodies are one of the major components of the ecosystem, which contribute to the regulation of the biogeochemical cycle for the existence of life. The freshwater ecosystem is significant not only for the biological existence of life but also for the livelihood and economics of human society. Today, it has become difficult to ensure sustainable exploitation of any water body due to overpressure and imbalanced anthropogenic involvement. Aquaculture is an essential need of society, but the release of pollutants like heavy metals and organic chemicals in waterbodies is also unavoidable in the present social structure in underdeveloped and developing nations. The sustained release of pollutants in waterbodies has caused adverse impacts on aquatic animals, particularly fish, which is a major source of protein for humans. It has been reported that bioaccumulation of heavy metals is a common practice in fish. Besides, altered physico-chemical parameters of waterbodies are also one of the major factors to create toxicity as well as oxidative stress among fish. (Rattan and Sylg 2007). In waterbodies involved with anthropogenic activities, both heavy metals as well as other altered physico-chemical parameters create oxidative stress and lead to damage to their nucleic acid, protein, and induce lipid peroxidation among edible fishes, causing major economic loss to society (Frenkel and Casel, 2007).

Exposure to oxidative sources not only causes genetic mutations and DNA damage but also alters gene expression, which can affect the genetic diversity and adaptability in fish. Besides genetic alterations, immunosuppression, metabolic disruptions, and endocrine alterations can compromise the health, survival, and reproductive stress in fish (John *et al.,* 1997). Under adverse conditions of waterbodies, hypoxia is also one of the factors in fish that can disrupt the antioxidant defense system of fish, reducing their ability to neutralize ROS and thereby leading to cellular damage (Richard *et al.,*2011, Fagernes *et al.,* 2017, Renshaw *et al.,*2014).

In the fish bodies, some defensive mechanisms neutralize the free radicals and reduce oxidative stress.

Various types of food-based components act as antioxidants, viz., vitamin C, E, beta-carotene, selenium, polyphenols, and coenzyme Q 10, are found in fish food supplements, which normally protect from the adverse effects of oxidative stress (Perez-Jimenez *et al.,*2010). Besides the above, the cellular components like Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx), Glutathione reductase (GR), Thioredoxin reductase (TrxR), and Glutathione S-reductase (GSTs) are major enzymatic antioxidants found in the body of fish, which play a major role in reducing oxidative stress and related damage.

Glutathione S-Transferase is a diverse group of enzymes involved in the conjugation of the antioxidant molecule glutathione (GSH) to a wide range of endogenous and exogenous compounds. It consists of two distinct domains: an N-terminal that binds to GST and a C-terminal that binds to the substrate to detoxify. The major classes of GST in vertebrates, including fish, are alpha, Mu, Pi, theta, zeta, sigma, kappa, lambda, and omega classes. The GST family helps in scavenging ROS, reducing lipid peroxidation (Board 2003), catalyzing the conjugation of glutathione to endogenous compounds, acting as an anti-apoptotic factor, and thus protecting the cellular system from oxidative stress caused by pollutants of water bodies.

In the present study, the authors have analyzed to understand seasonal variations in physico-chemical parameters of a pond involved in anthropogenic activities and tried to understand the oxidative stress among two major carps (*L. rohita* and *C. catla*) from the pond, to determine the ability of fish to sustain in adverse oxidative stress. The purpose of the study was to understand the responsible mechanism for the adverse impact on fish health and productivity, so that suggestive measures may be recommended for the protection of the ecosystem and aquaculturists.

MATERIAL AND METHODS

Study Site

Every living being encounters oxidative stress; fish are more vulnerable because their habitat is open aquatic bodies, which are prone to natural and anthropogenic interference. The present study aims to analyze the seasonal water quality of ponds of Kutailabhata (21.2376° N, 81.3224° E) of Durg, Chhattisgarh, India, and the estimation of oxidative stress in two economically viable major carp (*L. rohita and C. catla*) of the pond. The temperature is variable in different seasons, viz., summer (30 °C-40 °C), monsoon (20 °C-40 °C), post-monsoon (25 °C-35 °C), and winter (10 °C-25 °C).

Physico-chemical analysis of water

The water samples were collected during the monsoon, post-monsoon, winter, and summer seasons in the morning before 8 am. The physico-chemical analysis of water was performed considering pH, conductivity, turbidity, magnesium, fluoride, iron, chromium, nickel, copper, lead, mercury, dissolved oxygen, nitrate, phosphate, total alkalinity, total hardness, and calcium hardness. Biological oxygen demand, chemical oxygen demand, and total dissolved solids were measured following APHA (1998). All experiments were performed in replicates of three.

Oxidative stress measurements in *L. rohita* and *C. catla*

In each season, samples of both fish species (*L. rohita* and *C. catla*) were collected, and blood samples from the fish were collected from the caudal vein to measure oxidative levels using the following methods: DPPH, FRAP, and H2O2 scavenging assay.

2,2- diphenyl-1-picrylhydrazyl (DPPH) assay was measured following Brand-Williams *et al.,*1995 at 515nm, and it was calculated by applying
% of Antioxidant activity = {(Ac- As)) /Ac} x 100
Where;
Ac - Control reaction absorbance,
As - Testing sample absorbance

Ferric reducing antioxidant power (FRAP) was measured at 593 nm following Benzie *et al.,*1996 and was calculated by applying the formula.
FRAP value = (A sample- A blank/A standard) A standard x C standard
Where;
A sample - absorbance of a sample,
A blank - absorbance of the blank (without antioxidant),
A Standard - absorbance of the standard,
C Standard - concentration of the standard

Hydrogen peroxide scavenging assay (H_2O_2) assay was measured following Ruch *et al.,*1989 and was calculated by applying the formula-
Scavenging Activity = {(Ac – As)/ Ac} x 100
Where;
Ac - control reaction absorbance,
As - testing sample absorbance

Micronuclei test and Statistical validation of data

The micronuclei test was performed by slide methods, followed by microscopic observations on the blood of both fish in all seasons. The picture of micronuclei was also subjected to an AI tool for better observations. All the data collected after each physico-chemical analysis and oxidative stress analysis were subjected to t-test, Chi-square test, and one-way ANOVA test for validation and inference of findings.

RESULTS

The pH of the pond in the whole year was found to fluctuate between a minimum of 7.18±0.13 in the monsoon season to a maximum of 8.18 + 0.08 in the post-monsoon season, but overall fluctuation in the year was found non-significant (>p@ 5%). The turbidity, TDS, conductivity, alkalinity, and hardness was found minimum1.73± 0.94 NTV, 215 ±55.8mg/l, 328.7 ± 15.0

mg/l, 72.5± 1.13 mg/l, 97.0 ± 9.89 mg/l, and 20.49 ± 1.0mg/l, respectively in monsoon season and maximum 12.78± 3.16 NTV, 215.5 ± 35.5mg/l,358.3 ± 3.49 mg/l, 124.00 ± 32.8 mg/l, and116.0 ± 325.4 mg/l respectively in summer season and all fluctuations over the year was found significant (<p @5%). The calcium, magnesium, and fluoride were found to be minimum, of 20.49 ± 1.0mg/l, 10.72 ±2.2 mg/l, and 0.09 ± 0.01 mg/l in the monsoon season and a maximum of 34.07± 3.8 mg/l,7.35 ± 3.1 mg/l, and 0.23 ± 0.3 mg/l in the summer season, and the yearly fluctuations were found significant (< p@ 5%). Nitrates and sulfates were found at a minimum of 0.52 ± 0.2 mg/l in the monsoon season and 0.04 ± 3.4 mg/l in the summer season, respectively, but a higher concentration of nitrate 6.89 ±8.35mg/l and for sulfate 27.35 ± 2.2 mg/l was reported in the winter season, but again the annual fluctuation of both was found significant (< p@5%). The fluctuation in concentration of calcium, magnesium, and fluoride was found statistically nonsignificant (>p@ 5%), with and minimum of 20.49 ± 1.1 mg/l in monsoon, 7.35 ± 3.1 mg/l in summer, and 0.09 ±0.01 mg/l in monsoon season, respectively. The maximum concentration was reported as 45 ± 2.5 mg/l in summer for calcium, 14.36± 1.2 mg/l in winter for magnesium, and 0.23 ± 0.3 mg/l in summer for fluoride. Annual fluctuation in all four seasons for iron, chromium, nickel, copper, lead, and mercury was not found significant (>p@ 5%).

Overall, observations of maximum fluctuation in physico-chemical parameters towards the lower side were reported in the monsoon season, and towards the higher side in the winter season, except for a few instances. This may be considered a mildly polluted state of the pond for the annual period (Table 1).

Table 1: Seasonal physicochemical alterations in the pond of Kutailabhata

Parameter	Monsoon (Mean±SD)	Post-monsoon (Mean±SD)	Winter (Mean±SD)	Summer (Mean±SD)	Chi-square	Significance
pH	7.19 ± 0.19	8.18 ± 0.08	7.40 ± 0.40	7.19 ± 0.20	0.0324	NS
Turbidity (NTU)	1.73 ± 0.95	26.39 ± 5.85	32.21 ± 3.80	12.78 ± 316.57	20.9525	S
TDS (mg/L)	215.00 ± 55.86	262.50 ± 58.69	396.00 ± 280.01	215.50 ± 355.86	58.8979	S
Conductivity (µS/cm)	328.70 ± 15.13	348.00 ± 66.46	236.40 ± 189.79	358.30 ± 349.64	145.4451	S
Alkalinity (mg/L)	75.20 ± 1.13	84.55 ± 3.61	146.00 ± 93.34	124.00 ± 328.28	31.7838	S
Hardness (mg/L)	97.00 ± 9.90	137.00 ± 50.91	172.00 ± 90.51	116.00 ± 325.46	14.0860	S
Calcium (mg/L)	20.50 ± 1.42	30.65 ± 3.18	45.26 ± 15.35	34.07 ± 38.50	2.7827	NS
Magnesium (mg/L)	10.72 ± 2.72	9.61 ± 1.00	14.37 ± 12.70	7.35 ± 31.04	5.4619	NS
Chloride (mg/L)	40.46 ± 1.63	41.80 ± 0.14	112.44 ± 88.33	54.98 ± 38.49	34.5286	S
Fluoride (mg/L)	0.09 ± 0.01	0.20 ± 0.08	0.53 ± 0.21	0.23 ± 30.08	0.1051	NS
Nitrate (mg/L)	0.53 ± 0.12	0.58 ± 0.02	6.90 ± 8.39	2.70 ± 33.30	13.2947	S
Sulphate (mg/L)	17.60 ± 1.31	14.60 ± 0.39	27.36 ± 22.44	8.04 ± 34.26	15.5923	S
Free Residual Chlorine (mg/L)	0.015 ± 0.007	0.105 ± 0.134	0.025 ± 0.021	0.03 ± 30.28	0.0287	NS
COD (mg/L)	12.06 ± 1.18	18.07 ± 8.49	43.66 ± 29.37	11.68 ± 35.20	3.5331	NS
BOD (mg/L)	6.26 ± 1.18	8.95 ± 0.03	16.03 ± 8.19	4.05 ± 31.20	3.8506	NS
Iron (mg/L)	0.36 ± 0.09	0.42 ± 0.05	0.41 ± 0.39	0.28 ± 0.03	0.4272	NS
Chromium (mg/L)	0.02 ± 0.00	0.015 ± 0.007	0.02 ± 0.00	0.02 ± 0.00	0.0000	NS
Nickel (mg/L)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.0000	NS
Copper (mg/L)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.0000	NS
Lead (mg/L)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.0000	NS
Mercury (mg/L)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.0000	NS

S: Significant difference at P<0.05, NS: Not Significant, SD: Standard Deviation, Units for Calcium, Magnesium, Chloride, Fluoride, Nitrate, Sulphate, F.R. Chlorine, COD, BOD, Iron, Chromium, Nickel, Copper, Lead, and Mercury are in mg/L.. Conductivity is in µS/cm, and Turbidity is in NTU.

Under polluted water, a major disturbance is the generation of free radicals and the development of oxidative stress, and fish, a major biotic component of the pond water system, are very much vulnerable to oxidative stress, which leads to direct loss to the fishery industry and economy of concerned people. The authors have analyzed oxidative stress in two major carps, *L. rohita* and *C. catla,* which are most demanding from an economic point of view. Both carp were captured from the pond in all four seasons (monsoon, post-monsoon, winter, and summer), and their oxidative stress was analyzed following the DPPH method, FRAP method, and H_2O_2 method. *In L. rohita,* it was found maximum in winter (38.72 ± 15.82), and the minimum was found in the monsoon (18.49 ± 9.32). Annual oxidative stress and annual oxidative stress exposure following the DPPH method were found to be significant (F= 6.9208> p@ 5%) (Table no. 2).

Table 2: Seasonal variation in antioxidant assays (DPPH, FRAP, H_2O_2) for *Labeo rohita*

Fish ID	Season	DPPH (%)	FRAP (µmol Fe^{2+}/L)	H_2O_2 Scavenging (%)
L1	Winter	42.446	69.006	28.235
	Summer	45.529	43.026	18.824
	Monsoon	13.669	30.948	23.529
	Post-Monsoon	22.919	31.404	14.118
L2	Winter	48.510	56.700	18.824
	Summer	54.779	54.421	41.176
	Monsoon	27.030	11.121	1.176
	Post-Monsoon	32.682	17.958	17.647
L3	Winter	41.932	56.244	23.529
	Summer	32.169	55.789	7.059
	Monsoon	5.447	24.567	14.118
	Post-Monsoon	36.280	57.840	14.118
L4	Winter	49.640	60.119	16.353
	Summer	38.849	52.826	4.706
	Monsoon	27.544	25.706	28.235
	Post-Monsoon	41.418	5.652	18.824
L5	Winter	11.100	33.227	41.176
	Summer	47.585	24.339	29.412
	Monsoon	18.808	56.472	8.235
	Post-Monsoon	27.030	40.064	25.882
F-Value	Across Seasons	6.9208	2.9162	0.8323
Significance		*P < 0.05 (Significant)*	*P > 0.05 (Not Significant)*	*P > 0.05 (Not Significant)*

All values are expressed as % scavenging activity. *Labeo rohita* was assessed for antioxidant activity using three assays across four seasonal samples. Significance was tested using one-way ANOVA (F-test). P@5% denotes the significance level at $P < 0.05$. DPPH assay showed significant seasonal variation, while FRAP and H_2O_2 assays did not.

In *C. catla* also the antiradical activities measured through the DPPH method were found to be maximum in the winter season (44.39 ± 11.63) and the minimum was reported in monsoon (19.00 ± 12.357), and the annual fluctuations in antiradical activities were recorded as significant (F= 3.4029 > p@5%) (Table no. 3).

Table 3: Seasonal variation in antioxidant assays (DPPH, FRAP, H_2O_2) for *Catla catla*

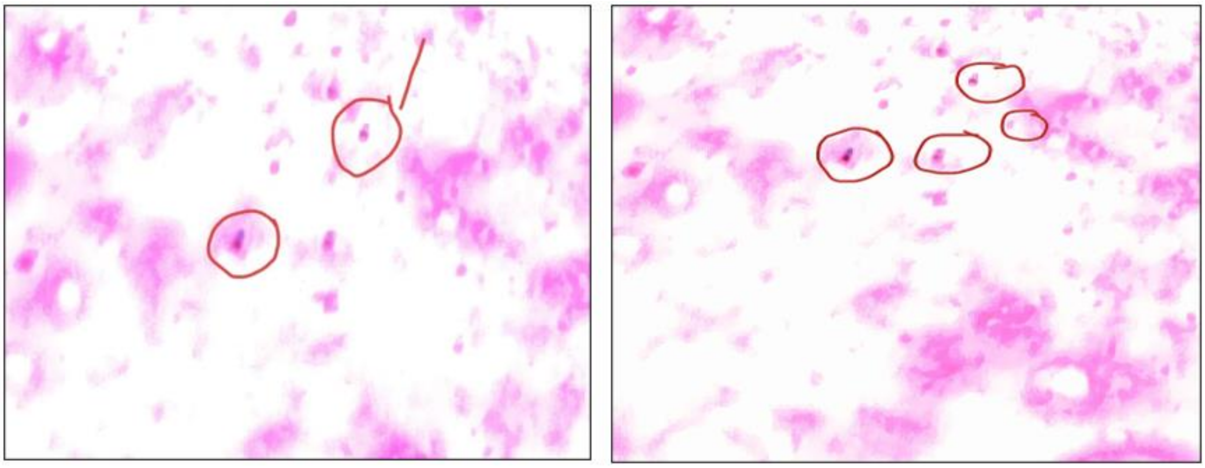
Fish ID	Season	DPPH (%)	FRAP (µmol Fe^{2+}/L)	H_2O_2 Scavenging (%)
C1	Winter	43.474	51.459	45.882
	Summer	17.266	23.200	37.647
	Monsoon	21.891	58.979	2.353
	Post-Monsoon	38.335	31.404	16.471
C2	Winter	59.918	40.975	5.882
	Summer	24.460	5.880	54.118
	Monsoon	14.697	26.162	–54.118
	Post-Monsoon	23.433	17.958	5.882
C3	Winter	51.696	44.622	23.529
	Summer	50.154	51.914	48.235
	Monsoon	32.169	25.934	24.706
	Post-Monsoon	14.697	57.840	9.412
C4	Winter	36.280	61.030	45.882
	Summer	41.932	53.965	14.118
	Monsoon	41.932	53.054	23.529
	Post-Monsoon	11.614	5.652	48.235
C5	Winter	30.627	60.346	38.824
	Summer	67.626	61.714	28.235
	Monsoon	26.002	22.744	–23.529
	Post-Monsoon	6.989	40.064	4.706
F-Value	Across Seasons	**3.4029**	**0.7681**	**3.5600**
Significance		*P < 0.05 (Significant)*	*P > 0.05 (Not Significant)*	*P < 0.05 (Significant)*

All values are expressed as % scavenging activity. *Catla catla* was assessed for antioxidant activity using DPPH, FRAP, and H_2O_2 assays across four seasonal samples. Significance was tested using one-way ANOVA (F-test). P@5% denotes the significance level at $P < 0.05$.

The antioxidant power was also measured in both carp by the FRAP method, and in *L. rohita,* it was measured maximum in winter (55.05± 13.326) and minimum in monsoon (29.75 ± 16.626), but overall, annually, the antioxidant power was not found to be significant in *L. rohita*. Similarly, the antioxidant power in *C. catla* was also found non-significant (F=0.7681<p@5%), but it was reported maximum during winter (51.68±9.041) and minimum in monsoon and post-monsoon season (37.37 ± 17.199) (Table no. 2 &3).

The hydrogen peroxide scavenging method was applied to trigger cellular oxidative stress, and its potency of intracellular antioxidant defense was also measured in both *L. rohita* and *C. catla*. In *L. rohita,* it was again recorded as highest in the winter season (25.61 ± 9.814) and lowest (18.11 ± 9.854) in the post-monsoon season, but again the overall fluctuation was not found significant (F= 0.83228 <P@5%) in *L. rohita*. In *C. catla,* maximum potency was recorded in summer (36.46 ± 15.928) and minimum was recorded in the post-monsoon season (16.95± 33.561), but the overall annual potency of intracellular antioxidant defense was found significant (F=3.56<p@5%) (Table nos. 2 and 3).

Banking on antiradical activities, antioxidant power and potency of intracellular antioxidant defense, a micronuclei test was performed to validate the impacts on fish and it was found that substantive micronuclei were developed in both *L. rohita* and *C. catla* but comparatively the intensity in micronuclei development in blood cell was found higher in *C. catla* than the *L. rohita*, thus proved that both fishes were get affected by fluctuating polluted condition of pond but *L. rohita* having higher tolerance limit in comparison to *C. catla* (Figures 1).



(a) **(b)**

Fig. 1: Blood film of (a) *Catla catla* and (b) *Labeo rohita* showing micronuclei at 100x magnification.

DISCUSSION

The water quality index is a mathematical mechanism for summarizing the water quality data into simple terms, e.g., Excellent, good, bad, etc., and reflects the level of water quality in rivers, streams, ponds, lakes, etc. The water quality class is defined depending on the measured physical, biological, and chemical parameters, besides the purposes for which water is used, such as for drinking, agriculture, sanitation, or industry. Also, the metal quality indices have been applied to assess the drinking water resources concerning metals. Nowadays, the release of free radicals by aquatic organisms under the stress of pollutants in water is one of the measure parameters to determine the quality of water and its use. Several pollutants cause oxidative stress in aquatic organisms and result in compromised health of aquatic organisms and the related aquaculture economy. In the present study, some parameters have shown significant annual fluctuations, and some are nonsignificant, but remarkably, metals like iron, chromium, nickel, copper, lead, and mercury were not found at a nonsignificant (>p@ 5%) level. Another notable finding is in general, the maximum concentrations of various parameters were reported in the winter season and the minimum in the monsoon season. The oxidative stress was also found to be maximum in the winter season and minimum in the monsoon season in both of the fish species, which is correlated with physicochemical fluctuations of the pond; even the antiradical activities, antioxidant power, and potency of intracellular antioxidant defense were found to be correlated. As a result of free radical impact damage to the nucleic acid of both species has been reported in the form of micronuclei, but remarkably, it was reported to be maximum in *C. catla* in comparison to *L. rohita,* which explains the higher tolerance level of impact of adverse physicochemical parameters of water in *L. rohita.*

Stress in biological systems can be triggered by a variety of physical, chemical, or psychological stimuli, all of which can lead to oxidative stress. Oxidative stress occurs when the body's ability to manage ROS production is overwhelmed, resulting in damage to cells and tissues. ROS are primarily generated through normal aerobic metabolism, but their levels increase dramatically in response to stress, environmental toxins, or infections (Geiszt *et al.,*1997). A particular form of stress, oxidative stress, arises when there is an imbalance between the generation of reactive oxygen species (ROS) and the ability of the biological system to neutralize these reactive intermediates (Halliwell & Gutteridge, 1999). Stress in biological systems can be triggered by a variety of physical, chemical, or psychological stimuli, all of which can lead to oxidative stress. ROS are primarily generated through normal aerobic metabolism, but their levels increase dramatically in response to stress, environmental toxins, or infections (Geiszt *et al.,*1997).

The GST gene family has evolved in fish to adapt to varying environmental conditions, particularly in polluted waters. The presence of diverse GST isoforms across species reflects an evolutionary strategy to cope with different pollutants. Fish-specific GSTs, such as the rho class, are unique to aquatic organisms and exhibit distinct detoxification abilities compared to their terrestrial counterparts. In Coho salmon, multiple classes of GST genes have been identified, including omega, pi, and rho classes. Espinoza *et al.,* (2013) reported nine subfamilies of GST genes in Coho salmon, including alpha, mu, pi, and rho classes, with rho-class GSTs being the predominant isoforms involved in detoxification.

Chen *et al.,* (2017) identified 27 GST genes in the common carp genome, representing several cytosolic GST classes, including alpha, mu, pi, rho, zeta, and omega. Interestingly, two novel classes of GSTs, omega and zeta, were discovered, suggesting that these genes may have evolved in response to the aquatic environment's unique toxicological challenges.

Studies on flatfish exposed to heavy metals like cadmium and mercury have revealed an upregulation of mu-class and pi-class GSTs in the liver and gill tissues (Kim *et al.,*2009). This upregulation was closely linked to the fish's ability to survive in highly polluted environments. The GST activity in flatfish gills was particularly high, suggesting that these enzymes play a crucial role in detoxifying waterborne pollutants before they reach internal organs. In the present study, it has been established that *L.rohita* has a good ability to counter the pollutant condition, probably by neutralizing oxidative stress influenced by aquatic pollutants, in comparison to *C. catla,* evidenced by the least number of micronuclei in tissue, which supports minimum cellular and nucleic acid damage and finally minimum chances of morbidity.

CONCLUSION

Biomonitoring to assess ecosystem integrity regularly is essential for the management of aquatic ecosystems. Besides physicochemical analysis, some biomarkers, such as oxidative stress markers, have proved to be meaningful indicators for the health and survival of aquatic organisms and aquaculture. The damage to proteins or modifications of different classes of chemicals and amino acids during oxidative stress can give rise to protein carbonyls and other harmful biomarkers. Various reports of oxidative stress in fish models in response to pollutants have suggested various enzymatic and non-enzymatic antioxidants as biomarkers of oxidative stress. In the present study, it has been concluded that oxidative stress influenced by the physicochemicals of waterbodies and related damage to tissue and nucleic acids finally results in the morbidity of edible carp and the economy of aquaculture. In the present investigation, *L. rohita* has proved better than C.*catla* for aquaculture in a specific physicochemical condition of the water body. It may be used by aquaculturists for a better economy.

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