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Assessment of genotoxicity triggered by ammonium phosphate sulphate in fresh water fish *Pseudoetroplus maculatus* and prominent role of oxidative stress exposed strains

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Abstract

The use of various chemical substances for agricultural and other sectors including the production of food resources has augmented in recent scenarios. The rapid and extensive use of such chemical elements may influence the life of other nontarget organisms as well as various aquatic resources. In this regard, here the authors have implemented the comet assay, blood and immunological analysis followed by the determination of excessive production of free radicals. The major findings of the current investigation clearly revealed that the chronic and acute strains are predominantly known to be exposed to the ammonium phosphate sulphate and exhibited strong damages in the genetic material. Furthermore, the immunological analysis of gut, muscle and gill tissues of *Pseudoetroplus maculatus* with ImmunoglobulinIgM, Complement compound 3 (C3) and Complement compound 4 (C4) also revealed the same pattern. The excessive production of free radicals along with significant variation in the blood cells of tested strains were also recognized as a principal consequence instigated by the ammonium phosphate sulphate in *Pseudoetroplus maculatus*. The comparison of observed results of genotoxicity, oxidative stress, immunological, and blood analysis at specific concentrations validated that the acute strains are more prone to exposure.

Keywords: Fish; Genotoxicity; Immunoglobulin; Environment; Pollutant.

Introduction

The use of synthetic compounds in the agricultural sector has been greatly increased today due to its advantages like high reduction of unwanted and harmful organisms thereby saving the crop within a short span of time and providing economic stability to the cultivator. In addition to this, pesticides and fertilizers enhance production, reduce food loss along with the chemicals properly manage vector-borne disease transmission both to human andother animals (Sharma et al. 2019) (Al-Ghanim 2012). However, the uncontrolled use of artificial compounds greatly affects the aquatic environment causing severe damage to living beings, especially to fish since they are extremely sensitive to the minute changes in water causing a sudden change in their behavior and ultimately thesecompounds threaten the aquatic environment and its stability (Ogueji et al. 2020) (Cheng et al. 2020) (Renick et al. 2016) (Bharti and Rasool 2021) (Kuo et al. 2010).

Due to the sensitive nature of fishes to toxic elements fishes were used as bioindicators in numerous aquatic biota worldwide and they were employed as model organisms in the evaluation of toxicological along with safety analysis of several chemicals (Hong et al. 2018). Over usage of pesticides and fertilizers not only threatens the life of aquatic living beings but also enhances pollution in many ways. Pollution caused by the synthetic compounds is of great concern since it adversely affects the non-specific organisms include human beings caused by the bioaccumulation process (Nicolopoulou-Stamati et al. 2016). Continuous exposure to pesticides and fertilizers gradually became a reason for the imbalance of the ecosystem as they get accumulate in soil and finally disrupt the food chain (Dar et al. 2016). It has been recorded from the previous study reports that approximately 50% of aquatic pollution arises from the agricultural sector by the utilization of pesticides and fertilizers (Gavrilescu et al. 2015).

Agriculture plays a significant role in India's economy henceforth it is very essential to take necessary requirements and modifications to balance the negative impacts caused by the synthetic products. The current study focuses on the genotoxicity of ammonium phosphate sulphate in the fish *Pseudoetroplus maculatus*. As per the collected reports of previous studies, it is clear that the overuse of chemical compounds leads to pollution and biomagnification but the present study concentrates on the level of its impact on the gene of fishes and whether it causes any genetic alteration caused by mutation. It was determined via Comet assay, we also conducted blood analysis along with oxidative stress. Recently the population of fish in Kerala was estimated to be low when compared to the previous years this may be because of the issue caused by the use of chemical constituents to enhance the production also to protect the crop from harmful organisms causing damages to the crops and thereby financial loss. Most of the paddy field borders were related to river sides which greatly became a reason for the emergence of chemicals used in agricultural plots.

METHODOLOGY

Sample collection and Dissection

The *Pseudoetroplus maculatus* were collected from Bharathapuzha River (10°37'29.5"N, 76°35'57.1"E) and proceeded for the treatment. Fishes were acclimated in a 50L cement tank filled with well water, 75% of the water is renewed daily. During acclimation fishes were proceeded towards a photoperiod of 12 hrs of light and 12 hours of darkness. The fishes were provided with commercial pellets as feed during this process. Dissection were doneusing 200 fishes and the dissected section was pooled for further analysis

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Genotoxicity

In order to analyse the damage caused to DNA and its impact in entire blood sample of fish, Single Gel Electrophoresis (Comet assay) technique was employed (Nandhakumar et al. 2011).

MATERIALS USED FOR THE STUDY

Entire blood sample of fish supplied by the client in EDTA vial, D – PBS (# TL1006 Hi Media), Lymphoprep solution (Cat No: 07801, Stem Cell Technologies), Fetal bovine serum (Cat No: RM10432, Hi Media), 50 ml centrifuge tubes (# 546043 TARSON), 10 – 1000µl tips (TORSON), Agarose (Cat No: MB094, Hi Media), Agarose low melting (Cat No: RM 861, Hi Media, Ethidium bromide solution (Cat No: 15585011, Invitrogen), Frosted Glass slides (Blue Star), Glass coverslips (24 x 60mm)- (Blue Star) were used for the current study.

EQUIPMENT USED FOR THE STUDY

The following equipment's were employed in order to carry out the study. Analytical weighing balance (RADWAG), Microwave oven (LG), Centrifuge (Remi: R-8C), Pipettes: 2-10µl, 10-100µl and 100-1000µl, Inverted biological microscope (CK X 415F, Olympus, Japan), Biosafety cabinet (HealForce, China), 37^0C Incubator with humidified atmosphere of 5% CO_2 (Healforce, China), Horizontal submarine Gel Electrophoresis Unit (BioBee, India), Magnetic stirrer (BioBee, India), $_p$H Meter (BioBee), LSM-880 Confocal live imaging system (Carl Zeiss, Germany), Software: ZEN Blue (Carl Zeiss), Image J/FIJI software, TriTek Comet Score 2.0.03.8 (TriTek corporation).

In order to attain uniform distribution of whole blood, provided blood sample of fish were stored in gyratory shaker for about 5 minutes. The health of blood was confirmed by three-part hematology analyzer. 1ml of whole blood was mixed with 1ml of 2% FBS prepared in PBS and added it into 1.5 ml of Lyphoprep medium. The mixture was added in a fashion that layer of blood forms on the top of 15ml test tube consist of Lymphoprep medium. Later the entire suspension was centrifuged at 3000rpm for about 30 minutes at RT and the Plasma was removed without disturbing the buffy coat with Lymphocytes. Collected lymphocytes was cleaned twice using 1x PBS to make it free from impurities. 1% Normal Melting Point Agarose (NMPA) was prepared and then melted in a microwave oven for 3 minutes. 1% NMPA was used to film the glass slide surface later it was allowed to dry and solidify at 37^0C for about 30 minutes. 60µl of 0.5% Low Melting Point Agarose (LMPA) was mixed in PBS containing 20µl of cell suspension taken in 1.5 ml microcentrifuge tube. It was combined thoroughly by pipetting and the mixture was dropped over NMPA layer on a slide. In order to obtain a uniform layer over the NMPA coat, coverslip was set conscientiously over the gel. The gel was then allowed to solidify at 4^0C in a refrigerator for 30 minutes. The coverslips were removed carefully when the cell suspension -LMPA layer is solidified and 75µl of LMPA was added in to the agarose gel and by avoiding the formation of air bubbles, fresh coverslip was kept over the gel mixture layer. It was again allowed to solidify at 4^0C in a refrigerator for about 30 minutes. The slides were immersed into the staining trough consisting of cold lysis solution after removing the coverslips carefully and refrigerated for 2 hours or overnight. The slides were then separated from the lysis solution after the lysis of cell at 4^0C and kept exactly perpendicular to both electrodes with agarose coated slide facing upwards in horizontal submarine gel electrophoresis system. Fresh cold electrophoresis buffer was used fill the electrophoresis tank until it completely covers the slide. To obtain unwind DNA strands the slides were allowed to stay in alkaline buffer for about 30 minutes. The power supply was turned on with 0.74V/cm and the electric flow was adjusted to 300mA for 30 minute. Slides were carefully raised from electrophoresis buffer conscientiously flooded using neutralizing tris buffer ($_p$H-7.4) for 5 minutes. Later the buffer was cleared using distilled water and the cleaning process was repeated using distilled water for about 3-5 times. 50µl of Ethidium bromide solution (100ug/ml) was employed to stain in order to visualize the comets under fluorescent microscope equipped with an extraction filter of 515-560nm and a magnification of 20x. And finally, the Comet Tail length, Tail moment, % Tail DNA was estimated with the help of Tritek Comet score software (ver-2.9.9.3.8). For each condition a minimum of 50 comets were calculated.

Blood, Immunological and oxidative stress analysis

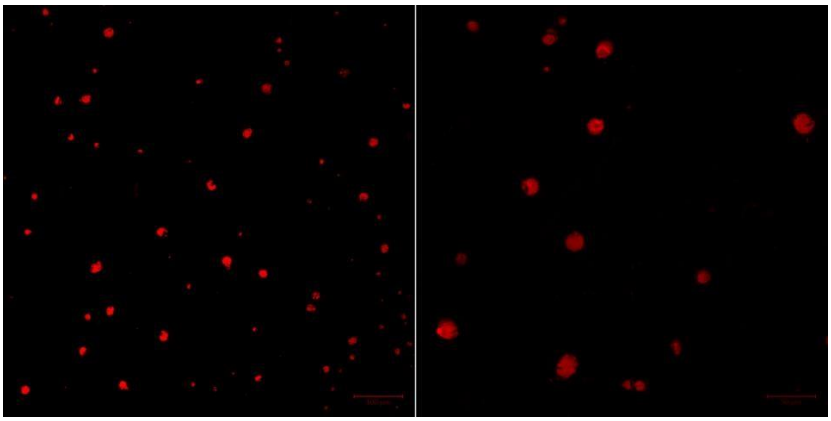
The method suggested by (Blaxhall and Daisley 1973) was used for blood and Immunological analysis. The following parameters were considered for blood analysis; WBC, Lym, Mid, Neu, Lym, Mid, Neu, NLR, PLR, RBC, HGB, HCT, MCV, MCH, MCHC, RDW-CV, RDW-SD, PLT, MPV, PDW-CV, PDW-SD , PCT, P-LCC, and P-LCR. The method suggested by (Prieto-Bermejo et al. 2018) with slight modification was used to determine the excessive production of free radicals.

Statistical analysis

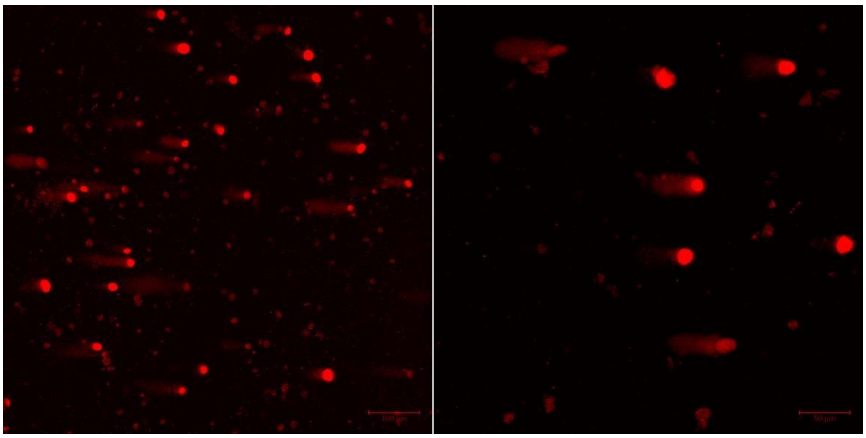
All the statistical analysis was performed using the SPSS version 27.0.0.

Results

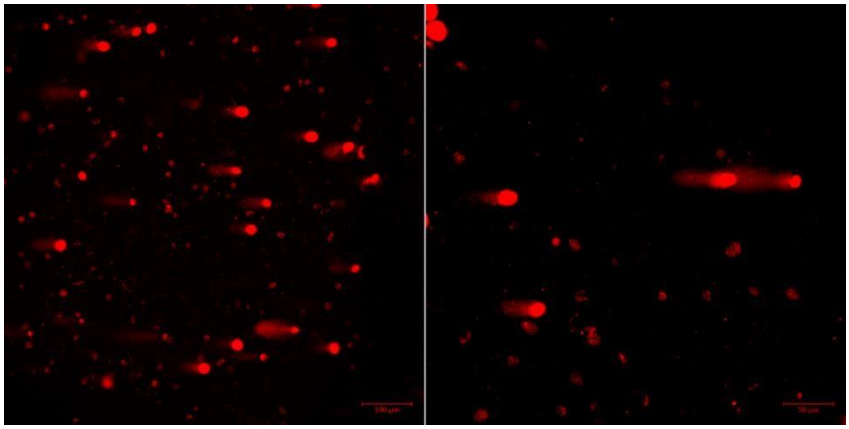
Effective genotoxicity was observed in Acute and Chronic cells with increased Tail moment, Comet length and Tail DNA (%) (Figure 1 and Figure 2). It was Because of the maximum range of toxicity instigated by the Ammonium Phosphate Sulphate in Chronic group, the reported group was found to exhibit increased Tail moment rather than others. As given in Fig. 1, the control group was found to exhibit moderate tail moment which revealed the fact that there exists no any genotoxicity/DNA fragmentation in the control, group. Altogether, the observed findings clearly confirmed that the Acute and Chronic conditions showed effective DNA damage and proved to be a genotoxic in nature. Table 1 depicted the Comet length, Tail DNA (%) and Tail moment (um) of Comets measured in different culture conditions of Control, Acute and Chronic conditions by TriTek Comet score software software. A minimum 50 comets were measured for each condition. The graphical representation of intensity towards Control, Acute and Chronic strains is given in Fig. 2.



A-Control group



B-Acute group



C-Chronic group

Figure: 1 Confocal images represented the genotoxic effect of Control, Acute and Chronic conditions of Fish whole blood cells by Comet assay at 10x and 20x magnification.

Blood analysis and Immunology

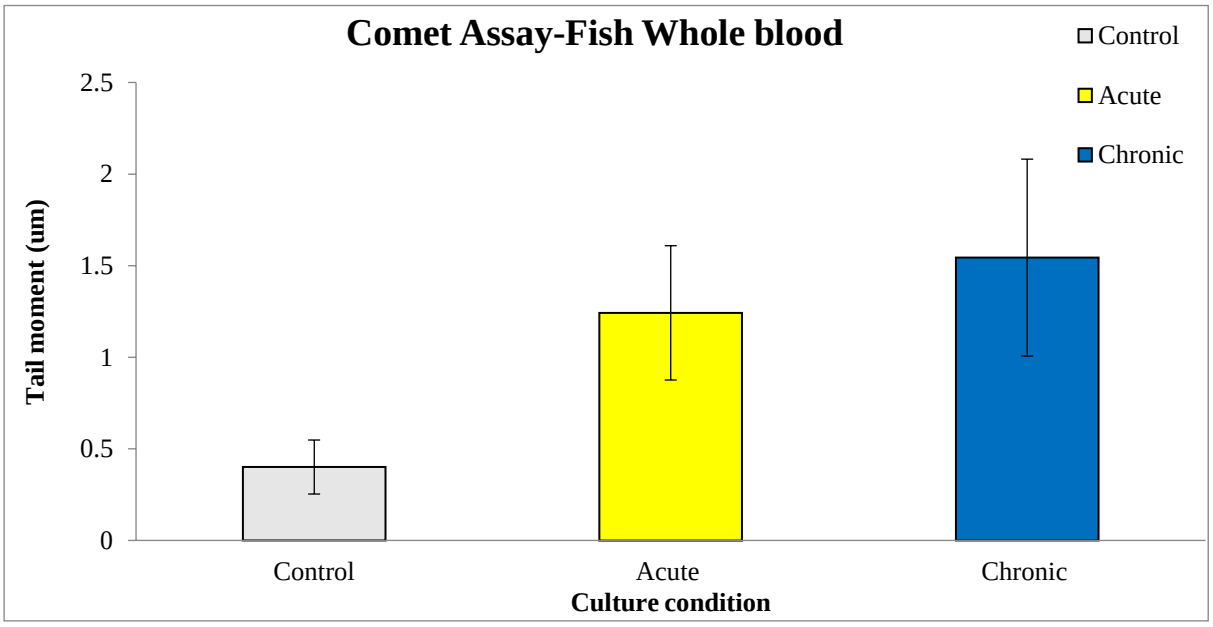


Figure 2 Tail moment of Control, Acute and Chronic conditions of Fish whole blood cells by Comet assay. Presented values were the average of greater than 50comets.

Immunological, Blood analysis and oxidative stress

The present study has also analysed the immunological inferences of the muscle, gill and gut of the selected specimen. The Immunoglobulin IgM, Complement compound 3 (C3) and Complement compound 4 (C4) (Table 2) have been considered in this strand. As found in the genotoxic responses, here the chronic strains were noted to express maximum values than the control strain in muscle and the observed range was found to be statistically significant when we conduct the paired test in the following manner; Chronic – Control and Acute – Control. Similar findings were observed for the gill and gut tissues with respect to the Immunoglobulin IgM Complement compound 3 (C3) and Complement compound 4 (C4). In addition to this, the current study has also analysed the various major constituents in the blood and found a strong variation of various selected strains of acute and chronic towards the control strains. Precisely, the there exits a prominent difference has been observed for the following parameters of acute And chronic strains towards the control groups; WBC, Lym, Mid, Neu, Lym, Mid, Neu, NLR, PLR, RBC, HGB, HCT, MCV, MCH, MCHC, RDW-CV, RDW-SD, PLT, MPV, PDW-CV, PDW-SD , PCT, P-LCC, and P-LCR (Table 3 (A-C)). The final part of the investigation hence ruled out the how the various strains were affected by the different intensities of oxidative stress. As given in Figure 3, the chronic strains were noted to posses highest degree of oxidative stress and it validated that the constant exposure towards the strain instigated

excessive production of free radicals in the target while the acute strains were known to have comparatively less amount of reactive oxygen species.

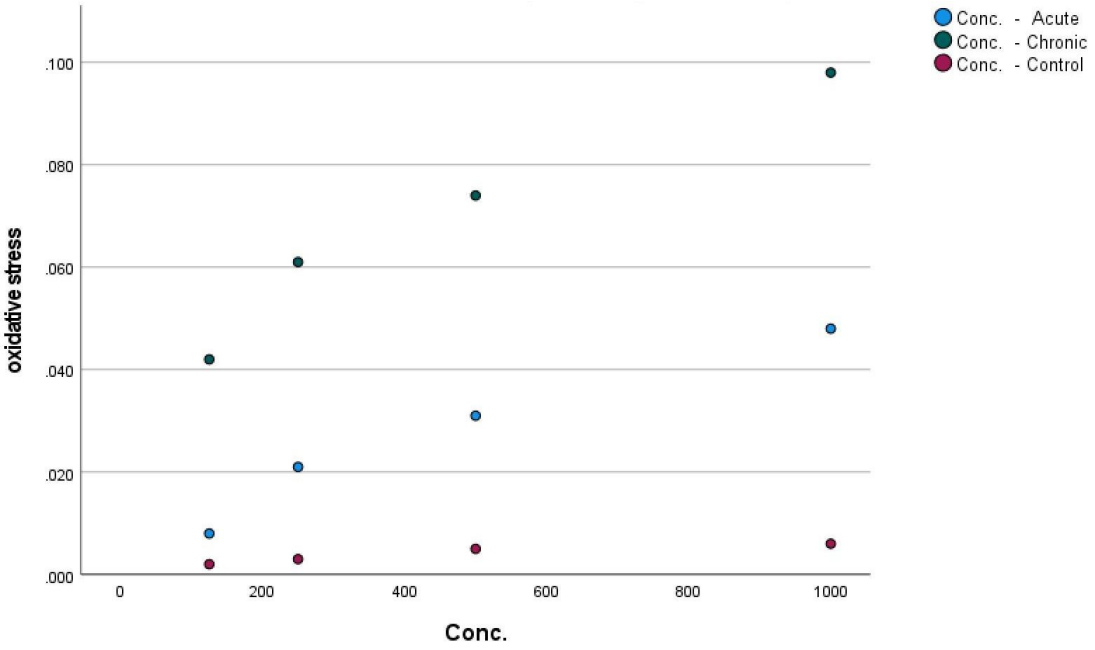


Figure 3 Oxidative stress and different strains

Table 1 Exposure towards the tissue

Culture condition	**Comet length**	**Tail DNA %**	**Tail moment (um)**
Control	27.41±7	9.27±5.63	0.4±0.33
Acute	56.7±21.90	13.26±6.28	1.24±0.82
Chronic	92.06±24.46	18.562±5.33	1.54±1.20

Table 2 Immunological analysis of the tested strains

Tissue		**Paired analysis**			
MUSCLE	Element	Chronic - Control		Acute - Control	
		T value	Sig.	T value	Sig.
	Immunoglobulin IgM	5.27	0.003	4.68	0.003
	Complement compound 3 (C3)	3.41	0.002	2.04	0.001
	Complement compound 4 (C4)	2.44	0.002	3.91	0.002
GILL	Immunoglobulin IgM	7.14	0.002	3.89	0.002
	Complement compound 3 (C3)	5.30	0.002	1.99	0.001
	Complement compound 4 (C4)	4.07	0.001	4.22	0.001
GUT	Immunoglobulin IgM	7.55	0.001	4.81	0.003
	Complement compound 3 (C3)	6.20	0.001	3.79	0.005
	Complement compound 4 (C4)	5.02	0.001	4.70	0.002

Table 3 Analysis for blood cells

A. Acute

Para	Result	Unit	Para	Result	Unit
WBC	79.17	10^9/L	MCV	*** *	fL
Lym	36.34	10^9/L	MCH	+++++	pg
# Mid	5.94	10^9/L	MCHC	+++++	g/dL
# Neu#	36.89	10^9/L	RDW-CV	** *	%
Lym%	45.9	%	RDW-SD	*** *	fL
Mid%	7.5	%	PLT	285	10^9/L
Neu%	46.6	%	MPV	8.1	fL
NLR	1.02	-	PDW-CV	14.4	%
PLR	7.84	-	PDW-SD	8.7	化
RBC	0.03	10^12/L	PCT	2.30	mL/L
HGB	58	g/dl	P-LCC	63	10^9/L
HCT	0.2	%	P-LCR	22.1	%

B. Chronic

Para	Result	Unit	Para	Result	Unit
WBC	46.18	10^9/L	MCV	*** *	fL
Lym	23.78	10^9/L	MCH	+++++	pg
# Mid	3.28	10^9/L	MCHC	+++++	g/dL
# Neu#	19.12	10^9/L	RDW-CV	** *	%
Lym%	51.5	%	RDW-SD	*** *	fL

Mid%	7.1	%	PLT	172	10^9/L
Neu%	41.4	%	MPV	7.4	fL
NLR	0.80	-	PDW-CV	13.6	%
PLR	7.23	-	PDW-SD	7.7	化
RBC	0.03	10^12/L	PCT	1.30	mL/L
HGB	4.5	g/dl	P-LCC	31	10^9/L
HCT	0.2	%	P-LCR	18.3	%

C. Control

Para	Result	Unit	Para	Result	Unit
WBC	55.96	10^9/L	MCV	*** *	fL
Lym	29.10	10^9/L	MCH	+++++	pg
# Mid	3.92	10^9/L	MCHC	+++++	g/dL
Neu#	22.94	10^9/L	RDW-CV	** *	%
Lym%	52.0	%	RDW-SD	*** *	fL
Mid%	7.0	%	PLT	253	10^9/L
Neu%	41.0	%	MPV	77	fL
NLR	0.79	-	PDW-CV	139	%
PLR	8.69	-	PDW-SD	8.2	化
RBC	0.02	10^12/L	PCT	1.90	mL/L
HGB	3.5	g/dl	P-LCC	49	10^9/L
HCT	0.1	%	P-LCR	19.2	%

Discussion

The recent years have witnessed to be reported with growing concern allied with the genotoxicity instigated by the various pollutants in water and land. Such circumstances would hostilely generate mutagenesis in various organisms including fishes. Because of the various roles possessed by the fishes in the environment, they were recognized to be suitable and efficient organisms for ecotoxicological investigations; bioaccumulation, heterotrophic web, xenobiotics activation, response to mutagens at low concentration and cytochrome P-450-oxidative metabolism (Çavaş and Ergene-Gözükara 2005). The impacts of pollutants or chemical elements in context with environmental bio-monitoring was reported in many previous studies (De Flora et al. 1993; Sanchez-Galan et al. 2001). Similar to their findings (Hayashi et al. 1998), assessed the aquatic organisms to rule out the intensity of genotoxicity while (Nagpure et al. 2008) reported the in-situ bio-monitoring analysis with genotoxicity assessment and yielded significant information concerning the augmenting pollution status of the aquatic resources. As mentioned by (Bajpayee et al. 2005), the comet assay, which has been used in the current study has recognized to be a reliable, sensitive and rapid approach and can be efficiently used for computing genetic damage in eukaryotic as well as prokaryotic cells. The possibilities of exploiting the comet assay for analysing the various substances from an industrial perspective including as pharmaceuticals, biocides, food additives and agrochemicals was reported in many instances including a study by (Brendler-Schwaab et al. 2005). Because of the advantages discussed in the above sections, the comet assay and fish erythrocyte analysis seems to be considered as a promising strategy in mutagenesis and environmental genotoxicity analysis. According to the major findings of the current study, there exits significant damage in genetic material which may probably originated through DNA adduct formations, DNA single-strand or double-strand breaks, DNA-protein cross-links and DNA-DNA cross-links, as reported by (Mitchelmore and Chipman 1998). The aforementioned study also reported that such interactions were initially linked with the toxic elements dissolved in the water resources. In addition to this, environmental pollutants are also known to modulate various defensive mechanisms and develop oxidative damage through excessively producing reactive oxygen species in aquatic organisms. The current study has hence uncoiled the intensity of oxidative stress and found that chronic strains are more predominant than the acute and control strains.

Previous studies reported that significant differences in the baseline values of DNA damage observed in various fish species indicated that various factors including species, age, and sex may influence the intensity of the impacts (Viganò et al. 2002). Furthermore, from the various observations made in this regard, it was clearly evident that *Pseudoetroplus maculatus* may be a sensitive fish, which may be rapidly exposed to the consequences by the

pollutant or the chemical. The immunological analysis performed with Immunoglobulin IgM. Complement compound 3 (C3) and Complement compound 4 (C4) showed that the chronic strains are more prone than others. Furthermore, among the various tissues tested, the gut region possessed relatively higher intensity than the gill and muscle. As mentioned earlier, the blood analysis also showed a great difference in various analysed parameters and the chronic strains are extremely exposed to the analysed chemical substance. The frequencies of the analysed blood cells were prominently varied when compared with the unexposed fishes. The findings of the current study emphasized the significance of investigating the impact of Ammonium Phosphate Sulphate and recommended its wider implications as an efficient biological marker of fish exposure to environmental pollutants and genotoxic elements.

Conclusion

The findings of this study concerning the impact of Ammonium Phosphate Sulphate towards the that *Pseudoetroplus maculatus* suggested a serious fear regarding the toxic nature of Ammonium Phosphate Sulphate when it is frequently exposed to fish, and indirectly to the human population too. The genotoxic studies through the comet assay verified the aforesaid circumstance and it was strongly supported by the immunologic, oxidative stress and blood analysis. Altogether, it was strongly reported that the finding could be used to develop efficient strategies to combat the threatening effects instigated by the chemical substances, especially the Ammonium Phosphate Sulphate.

There also exists a chance of ecological risk through possible bioaccumulation of the element and biomagnification over the food chain. Therefore for appropriate movements are advised in this regard to balance stability.

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DECLARATIONS

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not required

Availability of data and material

The data analysed during the present investigation are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Investigation, Methodology, Resources, Software, Roles/Writing - original draft, Formal analysis, and Visualization: Veena V

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