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Evaluation of Micronucleus Assay and Genotoxic Effect in *Oreochromis mossambicus* Exposed to a Fumaronitrile

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Abstract

Chemical contaminants of major concern in a polluted environment include persistent organic chemical, pesticides, oils, nutrients, heavy metals, etc. Some of these chemicals are known genotoxicants and can cause damage to the genetic material of the exposed organism. Apart from the enzymatic biomarkers, other parameters, such as DNA damage, should be evaluated for assessing the consequences of exposure of chemical. Damaged DNA may potentiate subsequent deleterious cellular events such as disease (e.g. cancer) and reduced reproductive competence. DNA damage in a variety of aquatic animals has been associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults. The DNA damage which is induced by fumaronitrile exposure could be evaluated by using the comet assay. The comet assay [alkaline single-cell gel electrophoresis] is a widely used technique to detect DNA damage due to environmental stress. The toxicological effect of fumaronitrile originates from its oxidizing action. Once entered in to the cell, in the presence of cellular reluctant, it induces DNA fragmentation, which is considered to be premutagenic change. The kind of the nuclear alterations observed in erythrocytes of *Oreochromis mossambicus* exposed to the fumaronitrile is binucleated cells, cells with bleb bed and lobed nuclei.

Keywords: Comet Assay, DNA Damage, Fumaronitrile, *Oreochromis mossambicus*

1. Introduction

Earth's ecosystem is being contaminated continuously by the different kinds of pollutants as organic, inorganic and the other forms. There are diverse types of pollutants that can contaminate the ecosystem. Some are persistent environmental degradation of chemical, biological and photolytic reactions exist for long time[1]. Most of the human activities that include domestic, agricultural or industrial have an impact on to pollute the biosphere. In the biosphere the soil, water and air are the most important things for the contamination however the water pollution, a major global environmental issue, has also assumed a serious threat to the sustainable development in India. World Health Organization statistics indicate that half of the India's morbidity is due to water related, Persistent Organic Pollutants (POPs) are considered as the silent killers due to their bio-accumulation. These are present everywhere in our environment including plants, animals and human beings. These are responsible for causing various lethal diseases and environmental problems. Water bore organism particularly the fish that can consume the POPs through polluted water, which can affect the food chain. Due to persistence, bioaccumulation potential and toxicity of many POPs in various sources of water have concern not only for the human welfare but also for the flora and fauna[2].

There are a lot of analysis to identify the contaminant that are present in the water sources however the

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Micronucleus (MN) test, that plays significant methodology to detect the modification occur in the genome level as well as the proteome level in the water born microorganisms and the animals. This type of analysis is simple and handled properly to find the modification occur in the DNA. The biomarkers are widely used to find the genome modification and the alterations that have crucial role and impact in producing the in the water born animals and affect the food chain. In our study we took fish as a model animal because it rarely requires the ethical committee approval. Fish respond to environmental toxic changes with adapting of their metabolite functions. These are present virtually in all environments and many species have been found to be susceptible for the environmental pollutants[3]. A comprehensive review of the acute and chronic toxicities of Pharmaceuticals and Personal Care Products (PPCP) that were dissolved in the water may affect the aquatic organisms[4].

MN is formed in the process of cell division and their expression can occur at times after the DNA damage event, depending on the cell cycle kinetics and the mechanism of induction. The MN assay, originally developed with mammalian species is today widely applied in fish and other aquatic organisms, including sea urchin, oysters, crabs and worms and in wild animals[5]. In addition, as compared to mammalian cells, it has been shown to be more sensitive for the induction of DNA damage however the DNA repair has been reported to be slower in fishes than other mammals[6,7]. Fishes are marvellous model animals for genotoxicological studies and provide early warnings for toxicants induced environmental alterations and degradations[8]. The fish biomarkers (DNA damage) were used as the effect of habitat contamination by genotoxic agents[3]. Furthermore, organic pollutants can induce the generation of Reactive Oxygen Species (ROS) such as Hydrogen Peroxide (H_2O_2), Superoxide (O_2) and Hydroxyl Radical (OH). This can cause enzyme inactivation and DNA damage[9].

Based on the above literature the present study was carried out in the North West regions of Tamil Nadu to identify the organic as well as the inorganic contaminants of different water sources. In these regions there is more amount of chemical sedimentation in the water logged places that can affect the aquatic flora and fauna. There are urgent need to concern about the environment in this place is crucial because of the hazardous accumulation of chemicals in the water sources. From the aquatic polluted source many inorganic chemicals and organic chemicals detected however the high amount of organic chemical in our region was identified as Fumaronitrile. Hence, in the study the organic chemical fumaronitrile was taken to find its toxicity in the in-vivo study. The significance on Fumaronitrile analysis on the aquatic organisms is the first report and nowhere reported. The toxicity analysis was performed at various concentrations and the identification of genome modifications was also performed using the aquatic organisms.

2. Materials and Methods

2.1 Sample Collection

The study area and sampling locations were identified based on the possible potential for water contamination by industrial effluents. The samples were collected from three areas for the present study. First sample site was the Kothapalli channel which is flooded with the industrial waste water from Hosur, Tamil Nadu. The second sample site, Kelavarapalli Dam receives the water source from Kothapalli channel and the third sample site Thenpennai river, is the outlet of Kelavarapalli Dam (Figure 1).

2.2 Water Quality Analysis

Various physico-chemical parameters such as temperature, pH, Dissolved Oxygen (DO), Salinity, Alkalinity, Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of water samples collected in situ from all three sampling stations were analyzed using standard methods (APHA, 1989)[10].

2.3 Atomic Absorption Spectrometry (AAS)

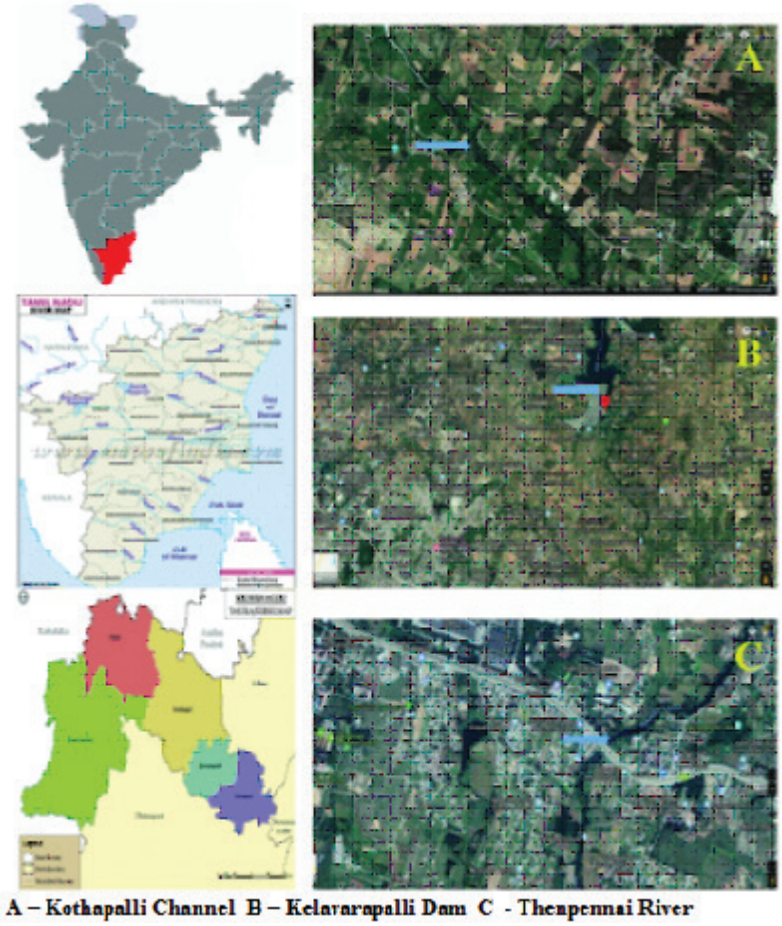
The concentration of residual metal ions present in the collected water samples from three different sites was quantified using Atomic Absorption Spectrophotometer according to method 3050B of EPA (Environmental Protection Act)[10].

2.4 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis of collected water samples was performed to detect the persistent organic pollutant. Wastewater samples were typically collected into amber flasks which are pre-cleaned by solvent. Samples were stored at low temperatures (4 to 20°C) prior to the analysis.

2.5 Experimental Procedure

Mature males and females *Oreochromis mossambicus* fishes were obtained from Veterinary University Training and Research Centre, Dharmapuri Dt., Tamil Nadu and maintained in the well aerated aquarium separately and intended for breeding within a week time. The laboratory temperature, 27 ± 0.5°C and normal illumination (approx 12 h light and 12 h dark) with the fish pellet twice a day were ideal. The following physicochemical characteristics: temperature 27 ± 0.5°C, pH 6.3 ± 0.4, Dissolved oxygen



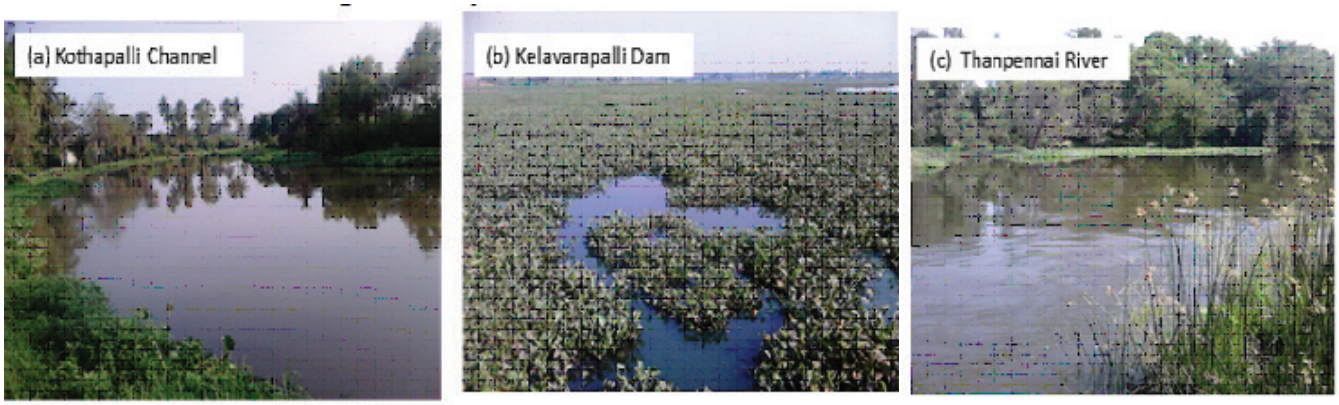


Figure 1. Study area.

6.5 ± 0.4 mg/L, salinity 0.6 ± 0.03 ppt, nitrite 0.04 ± 0.008 mg/L and hardness (as $CaCO_3$), 19.2 ± 0.05 mg/L. These parameters were measured according to the experimental procedures described.

The newly hatched juveniles/fries were separated from their respective mother and maintained in 300 L Fiber Reinforced Plastic (FRP) tanks and were maintained at an ambient, controlled temperature of 28 ± 2°C under natural photoperiod. A total of 200 fry (0 day old fry) were separated and used in four equal treatment groups. Fifty individual fries in each group were exposed to three different concentrations (1/10th of LC_{50}, 6 ppb, 1/20th of LC simultaneously. They were used for the experiment in the same day hatching. The young ones are fed with commercial granular feed throughout the experimental period.

2.6 Micronucleus Assay

Blood (1 mL) samples from different concentrations of Fumaronitrile exposed (90 days) fish was collected from the caudal vasculature using heparinised needle. The samples were stored in eppendorff tubes and kept in ice until centrifugation to separate the blood serum from the plasma. Centrifugation was done at 6000 × g for 2 min. Aliquots of 100 µL were transferred into 0.5 mL eppendorff tubes and samples were stored in the freezer at –80°C until assay was performed. The microscopic slides were prepared by smearing one drop of blood on clean glass slides, fixed in methanol for 10 min and air-dried at room temperature. The slides were transferred to 1N HCl at 60°C for 30 min and were air dried and placed in pre-chilled 1N HCl for 30 min. The slides were air-dried for overnight and stained with 5% Giemsa iodine solution for 10 min. Followed by gentle water wash, the slides were dried and observed under bright field light microscope. A total of 1000 erythrocytes were examined for each specimen to score the micronuclei[11].

2.7 Extraction of DNA

Volume of the reagents originally mentioned were customized and followed in this method with other modifications[12]. The stored and labelled Tissue sample of 20 mg was placed in 800 µl of buffer (10 mM Tris-HCl, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M Urea) and 10 µl of RNase (10 mg/ml) was added and homogenized and then incubated at 42°C for 1 h. After that 10 µl of Proteins K (10 mg/ml) was added and maintained at 42°C overnight. 800 µl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) was added to the micro centrifuge tube containing the mixture which was then inverted for 15 minutes. The micro centrifuge tube was then centrifuged for 15 min at 10,000 rpm and top aqueous layer was recovered. The DNA was precipitated in 1 M NaCl and two volumes absolute ethanol by centrifugation in 10,000 rpm. The DNA was washed with 70% ethanol; air dried and was resuspended in 60 µl of nuclease free water.

2.8 DNA Fragmentation

The extracted DNA was made run on 1% agarose gel. The DNA thus separated was visualized in UV light using fluorescence of ethidium bromide by direct comparison with a control. The fluorescence was documented using Gel documentation system.

3. Results

3.1 Water Analysis

Nowadays Hosur, the rich industrial hub of Krishnagiri (Dt.), Tamil Nadu is getting polluted by industrial effluents which is being released to nearby water streams. Due to that, aquatic organisms in the water bodies are being affected by these effluents and results in adverse effects to human beings and the environment. In the present study, water samples from three locations such as Kothapalli Channel, Kelavarapalli Dam and Thenpennai River, Hosur, Krishnagiri (Dt.) were collected, and they were subjected to physico-chemical (Table 1). AAS and GC-MS analyses to determine the probability of any toxic compounds.

Persistent Organic Pollutants (POPs) are considered as the silent killers due to their bio-accumulative and long persistent natures. These are present everywhere in our environment including plants, animals and human beings. The water sample analysis was performed by standard method further analysis was carried by GC-MS. GC-MS results showed pyrazine, a heterocyclic aromatic organic compound predominantly in all three sampling sites. This may be due to the high use of pyrazine and its derivatives in pharmaceutical industries for their wider applications like antitumor, antibiotic and diuretic activities. Next to pyrazine, fumaronitrile was the major compound found in GC-MS analysis (Table 2).

Further analysis was performed on the concentrations of trace metal elements such as Copper (Cu), Lead (Pb),

Table 1. Physiochemical parameters of water samples collected from the study area

S. No.	Parameters	Unit	Study Area		
			Kothapalli Channel	Kelavarapalli Dam	Thenpennai River
1.	Temperature	°C	27 °C	27 °C	27 °C
2.	Total dissolved solids	mg/l	784.0	756.0	758.0
3.	Total suspended solids	mg/l	6.0	4.0	4.0
4.	pH @ 25°C	-	7.83	7.62	7.66
5.	Dissolved Oxygen	mg/l	6.20	4.8	5.90
6.	Alkalinity	mg/l	435.0	390.0	395.0
7.	Dissolved Carbondioxide	mg/l	36.32	31.61	3.58
8.	Biological Oxygen Demand (3 Days @27°C)	mg/l	5.80	4.0	4.0
9.	Chemical Oxygen Demand	mg/l	44.80	41.60	41.60
10.	Phosphate as PO_4	mg/l	1.06	0.93	0.73
11.	Nitrate as NO_3-N	mg/l	0.71	0.72	2.31
12.	Silica as SiO_2	mg/l	23.12	27.26	24.61
13.	Iron as Fe	mg/l	0.08	*BDL [*DL - 0.05]	*BDL [*DL - 0.05]
14.	Sulphate as SO_4	mg/l	40.35	18.37	22.63
15.	Calcium as Ca	mg/l	72.45	88.55	76.47
16.	Magnesium as Mg	mg/l	24.09	11.88	28.94
17.	Chloride as Cl	mg/l	168.25	178.15	183.10
18.	Sodium as Na	mg/l	408.80	419.17	413.98
19.	Potassium as K	mg/l	37.82	34.71	29.53
20.	Fluoride as F	mg/l	0.96	0.99	0.96

*BDL – Below Detectable Limit.
*DL – Detectable Limit.

Zinc (Zn) and Nickel (Ni) present in the water samples were analysed through Atomic Absorption Spectrometry (AAS) and was tabulated in Table 2. The sampling site Kelavarapalli Dam has showed high concentrations of Cu (0.0475 ppm/unit) followed by Zn (0.0286 ppm/unit) whereas Pb (0.1882 ppm/unit) and Nickel (0.0203 ppm/unit) observed in high concentration level at the sampling site Thenpennai River (Table 3).

Chronic toxicity study was carried out by taking fifty individual fries in each group were exposed to three different concentrations (1/10 th of LC_{50}, 6ppb, 1/20 th of LC_{50}, 3 ppb and 1/30th of LC_{50}, 2 ppb) of fumaronitrile and control also maintained simultaneously. They were used for the experiment in the same day hatching. The young ones are fed with commercial granular feed throughout the experimental period.

3.2 Micronucleus Assay

Micronucleus assay was performed on the erythrocytes of *Oreochromis mossambicus* exposed to fumaronitrile. The frequencies of micronuclei, observed in erythrocytes of *Oreochromis mossambicus* showed differences among the tested three concentrations. The kind of the nuclear alterations (Figure 2) observed in erythrocytes of *Oreochromis mossambicus* exposed to the fumaronitrile are binucleated cells, cells with blebbed and lobed nuclei. The control test also showed nuclear alterations similar to those found in fumaronitrile tests, but in lower frequency.

3.3 DNA Fragmentation Assay

In the present study, the investigation was made to observe the fumaronitrile induced DNA damage. After

Table 2. Major compounds detected in water samples collected from the study area

S. No.	Compounds	Study Area		
		Kothapalli Channel	Kelavarapalli Dam	Thenpennai River
1.	Dodecyl isobutyl carbonate	++	+	–
2.	Trichloroacetic acid, pentadecyl ester	++	+	+
3.	1H-Indene, 5-butyl-6-hexyloctahy	++	–	–
4.	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol	++	–	–
5.	Spiro[2.3]hexan-4-one, 5,5-diethyl-	++	–	–
6.	Decane, 1,1'-oxybis	++	+	+
7.	Undecane, 5-methyl-	++	+	+
8.	Oxalic acid, 6-ethyloct-3-yl ethyl ester	++	+	+
9.	Fumaronitrile	++	+	+
10.	Methoxy acetic acid, decyl ester	++	–	–
11.	4-Methyldocosane	++		–
12.	1-Cyclohexene, 1-ethynyl-	++	+	–
13.	Pyrazine	++	++	+
14.	Cyclopentene, 1-ethenyl-3-methylene-	++	+	+
15.	Propanedinitrile, methylene-	++	–	–
16.	Tridecane, 1-bromo-	++	+	+
17.	Bicyclo[2.1.1]hex-2-ene, 2-ethenyl-	++	+	+

Table 3. Concentrations of elements present in water samples collected from the study area

Concentration of Elements (ppm)	Study Area		
	Kothapalli Channel	Kelavarapalli Dam	Thenpennai River
Copper (Cu)	**0.0007**	**0.0475**	**0.0054**
Lead (Pb)	**0.1739**	**0.1597**	**0.1882**
Nickel (Ni)	**0.0152**	**0.0051**	**0.0203**
Zinc (Zn)	**0.0024**	**0.0286**	**0.0041**

exposure of *Oreochromis mossambicus* with different concentrations of fumaronitrile, liver cell DNA was isolated. This isolated DNA was made run on 1% agarose gel and the pattern of DNA fragmentation was observed under a UV transilluminator. Chromosomal DNA was detected on the upper portion of the gel in control fish sample. The results confirm that DNA fragmentation was detected in DNA extracted from cells treated with fumaronitrile (Figure 3a, 3b), whereas no fragmentation of DNA was observed in the control group. The main mechanism of action of fumaronitrile for the induction of cytotoxicity and genotoxicity is probably due to an increase in oxidative stress. As shown in result, there was an elevated DNA fragmentation in the liver of *Oreochromis mossambicus,* associated with increased ROS response and consequent oxidative stress due to fumaronitrile which can be responsible for the observed DNA damage.

4. Discussion

GC-MS is becoming the tool of choice for tracking organic pollutants in the environment. In the present study, GC-MS results showed pyrazine was found predominantly followed by fumaronitrile in all three sampling sites. This may be due to the high usage of these compounds in pharmaceutical industries for their wider applications like antitumor, antibiotic and diuretic activities. In recent years monitoring of emerging contaminants

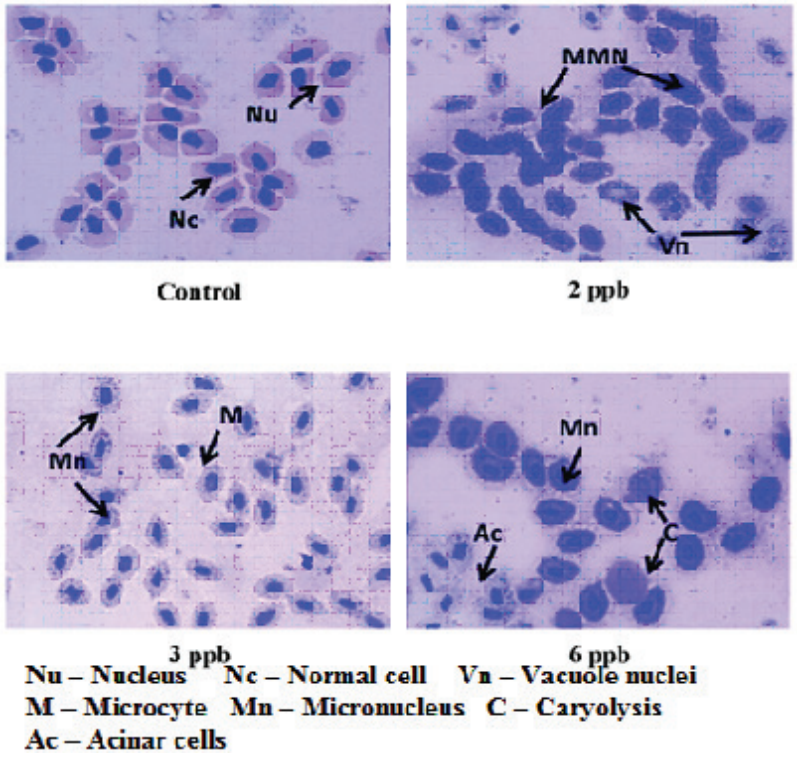


Figure 2. Micronucleus assay of erythrocytes of *Oreochromis mossambicus* exposed to different concentration of fumaronitrile.

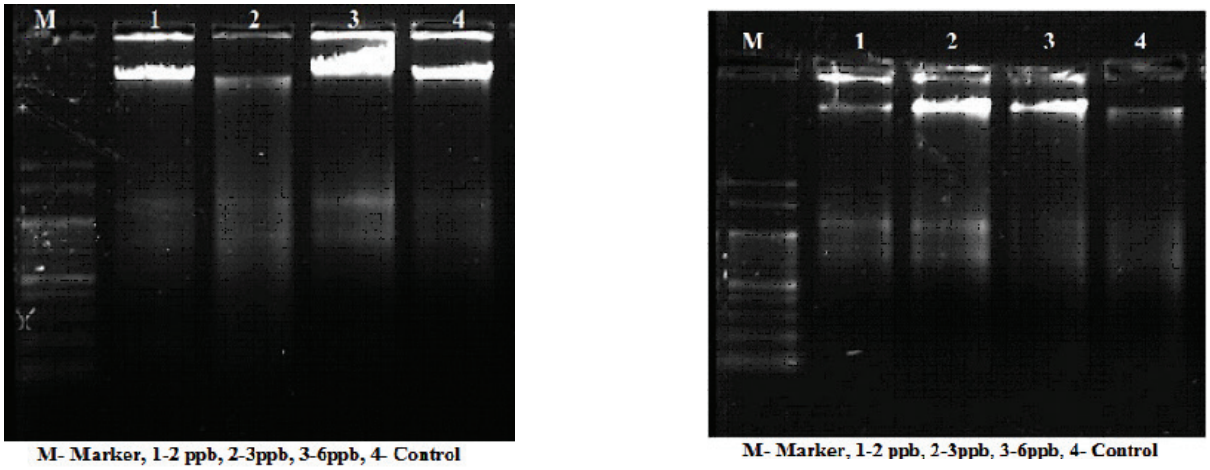


Figure 3. a. Fumaronitrile induced DNA fragmentation in liver of male *Oreochromis mossambicus.* b. Fumaronitrile induced DNA fragmentation in liver of female *Oreochromis mossambicus.*

such as PPCPs in Indian rivers has been taken up by many researchers[13,14]. In GC-MS analysis distribution of organochlorine pesticides in Indian marine environment was shown by several authors[15]. There are no reports on aliphatic nitrile compounds fumaronitrile used on the aquatic organisms hence this is the first report on the compound taken in this study. The major pharmaceutical and industrial effluents discharged which is the leading ecological risk to resident organisms and humans. Being no literature found for toxicity and effects of fumaronitrile on fresh water fish, fumaronitrile has been chosen for further study.

Molecular markers of the biological effects of contaminants on organisms (Biomarkers) can be used as diagnostic and prognostic early warning tests to detect and assess the effects of pollution, particularly low concentrations of complex mixtures of contaminants, on environmental quality[16]. In the present study, genotoxicity are evaluated as biomarkers to determine the effect of fumaronitrile on *Oreochromis mossambicus*. The micronuclei and DNA fragmentation assay, is widely used to evaluate the genotoxicity of fumaronitrile on *Oreochromis mossambicus*. Our results point out that the DNA damage in liver of *Oreochromis mossambicus* increases with increasing fumaronitrile concentration, indicating that fumaronitrile-induces DNA damage in *Oreochromis mossambicus* and the levels of micronuclei formation imposed by fumaronitrile could be quantified by micronuclei assay.

Fragmentation of DNA in the form of a ladder due to endonucleolytic attack is reportedly considered as an apoptosis and DNA damage related event. DNA fragmentation was considered as one of the later steps in apoptotic program. DNA ladder formation is observed in liver of *Oreochromis mossambicus* exposed to fumaronitrile[17]. The present study with *Oreochromis mossambicus* could confirm the genotoxicity of fumaronitrile, which is evaluated by the evidence of fragmentation of the genetic material. These results support the finding of who stated that atrazine herbicides induce DNA fragmentation in different organisms. Moreover, DNA fragmentation is evidenced in the present study by DNA gel electrophoresis in Atrazine treated *Poecilia sphenops* liver.

The toxicological effect of fumaronitrile originates from its oxidizing action. Once entered in to the cell, in the presence of cellular reductants, it induces DNA fragmentation or DNA strands breakage, which is considered to be premutagenic change. The DNA fragmentation or DNA strand breaks are also considered to be a kind of lesion that is potentially premutagenic; the production of breaks in the DNA strands being related to mutagenic and carcinogenic properties of chemicals[18,19].

The induction of DNA damage in *Oreochromis mossambicus* with significant effects from fumaronitrile concentration indicates potential genotoxic properties of fumaronitrile. The results of present study are in agreement with the findings of[20] for the erythrocytes of *T. mossambica* after exposure of monocrotophos. Diclofenac has known toxic and genotoxic effects in non-target organisms, like fish, where it can bio accumulate and change cellular reactions in liver, kidney and gills. It was reported that Diclofenac induces oxidative damage to the genetic material such as DNA[21]. The drug can directly produce DNA damage by means of the creation of adducts without significant ROS production[17]. Domoic acids is a naturally occurring toxin produced by algae, specifically the diatom species *Pseudo-nitzschia*[22]. Recently, reported that domoic acid induce DNA fragmentation in Caco-2 cells[23,24].

Currently nanoparticles are the most commonly used nanomaterial. Silver nanoparticles are a genotoxic agent that induces DNA double strand breaks. Both in vivo and in vitro studies provide evidences that engineered nanomaterials induce genotoxicity in rainbow trout, zebra fish[25–29]. The DNA damage can be correlated with the clastogenicity of the mutagen because an increase in both micronuclei and DNA migration due to environmental mutagen has been reported in fishes[30].

The main mechanism of action of fumaronitrile for the induction of cytotoxicity and genotoxicity is probably due to an increase of oxidative stress. As shown in Figure there is an elevated DNA fragmentation in the liver of *Oreochromis mossambicus,* associated with increased ROS and the consequent oxidative stress due to fumaronitrile which can be responsible for the observed DNA damage. This result is supported by the finding of[32] who stated that nanoparticles induce cellular damage through ROS production and also cause genetic damage. The DNA fragmentation in fish tissues evidence that nanoparticles induce DNA damage. Further the genotoxicity effect of fumaronitrile in *Oreochromis mossambicus* was confirmed by micronuclei studies.

The micronucleus test in peripheral erythrocytes provides a possible approach to monitor the effects of environmental genotoxic agents in fish. The micronucleus assay in erythrocytes of fish was widely used as a marker for environment pollutant. The chemicals cause significant damage in the red blood cells of *Channa punctatus* even at a lower concentration[31–33].

Genotoxicity of fumaronitrile chemical for all tested concentrations can be verified by the results which is significantly different from the control test. The highest concentration of fumaronitrile (6 ppb/L) exposed to *Oreochromis mossambicus* shows the highest rates of cells with micronuclei, indicating the high mutagenicity of the fumaronitrile. The result of present studies suggests that there is high frequency of micronuclei and nuclear abnormalities in *Oreochromis mossambicus* exposed to the different concentrations of fumaronitrile. Campos Ventura[34] also observed that atrazine induce high rate

of micronuclei frequency and nuclear abnormalities in *Oreochromis niloticus* (Perciformes, Cichlidae). Cavas[35] also reported that commercial formulation of atrazine treatment significantly induces micronucleus formation in goldfish (*Carassius auratus*). According to[36] on exposure to different concentrations of glyphosate there is an increase in the frequency of micronuclei in erythrocytes. In Nile tilapia (*Oreochromis niloticus*), exposure to a textile mill effluent for 9 days causes an increase of micronucleus frequency in gill. Elevated levels of micronucleus frequency are also detected in flounder (*Platychthys flesus*) after exposed to oil spill from Butinge oil terminal in the Baltic Sea[37,38].

5. Conclusion

The finding of the present study demonstrates that fumaronitrile possess DNA damaging potential in *Oreochromis mossambicus.* DNA fragmentation and the micronucleus test as genotoxicity endpoints and reported significant increases in the frequencies of both micronuclei and DNA damage following exposure to fumaronitrile. These findings may provide biomarkers for fumaronitrile which induce genotoxicity that could be useful for investigating the effects of toxic blooms on wild fish populations.

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