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Environmental biomonitoring using different biomarkers and their contribution in aquatic environmental research

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Abstract
In an era where innumerable biological and synthetic compounds are perpetually introduced into the market every day in various forms, such as pharmaceuticals, cosmetics, nutraceuticals, fertilizers, pesticides, and nanoparticles, to serve multiple purposes, there is a need to develop rapid screening methods to evaluate their biosafety. Wide array of biomarkers used to demonstrate exposure to and effect of environmental contamination. In order to assess exposure to or effect of environmental pollutant on aquatic environment the following suite of fish biomarkers may be examined, oxidative stress parameters, hematological parameters, immunological parameters, genotoxic parameters and histology. The text explores the latest knowledge and thinking on these very important approaches for the assessment of environmental health, management and conservation**.** This review focuses on measurement of the genotoxic potential of chemicals in aquatic organism at different level of biological organization under field and laboratory conditions by using comet assay, micronucleus assay, polychromatic erythrocyte count.

Keywords: biomonitoring, comet assay, micronucleus assay, repair, polychromatic erythrocyte frequency, normochmatic erythrocyte frequency

Introduction
Human and other animals are exposed to a variety of contaminants that are present in the environment due to anthropogenic activities. Contaminants can be incorporated in the organism via different routes and get bioaccumulated in different tissues and can induce variation in cellular or biochemical components, processes, structures or functions. To assess the quality and health of an environment, use of a biological community is called as environmental biomonitoring which can clearly reveal early responses to environmental stress. Numbers of biomarkers are used in a routine biomonitoring program of aquatic environment in order to evaluate the pollutant induced stress syndrome. These include behavioral response, genotoxicity (comet assay, MN assay, sister chromatid exchange (SCE), chromosomal aberrations, polychromatic erythrocyte frequency), hematology and histopathological alterations. Fish respond to toxic agents similar to higher vertebrates and can allow the assessment of substances that are potentially hazardous to humans. Anthropogenic activities such as industrial, agricultural, domestic and urban lead to accumulation of toxicants in water bodies. These genotoxins are capable of damaging the DNA and show the irreversible effect. The importance of detection of genotoxic risk associated with polluted water along with the importance of comet assay and a micronucleus assay in environmental health assessment was given by Osman, (2014) [69]. This section includes the detailed review of literature highlighting the important studies using the biomarkers like micronucleus assay, comet assay and polychromatic erythrocyte frequency to study the effect of different xenobiotics. A detailed study was conducted on the relevance of genotoxicity tests in aquatic environment research.

Micronucleus Assay
Schroedter, (1966) [78] discovered micronuclei in bone marrow after treatment with enzymes. The method has been developed and expanded to a wide range of genotoxicity testing. Schmid, (1976) [80] used micronuclei as a parameter for the first time.

The micronuclei test (MNT) has been widely used as a fast method of testing genotoxic agents in various mammalian models (Sehgal *et al*., 2011 and Garg *et al*., 2008) [38]. Micronucleus test has been used by a number of researchers for assessing the genetic damage (Ansari *et al.*, 2011; Galindo *et al.*, 2014; Kumar *et al.*, 2013; Nwani *et al.*, 2011) [13, 37, 50, 66]. Several scientists have also identified other nuclear abnormalities, including nuclear bud, fragmented nucleus, vacuolated nucleus and binucleated cells as an indicator of genotoxicity (Ayllon and Garcia-Vazquez, 2000; Cavas and Ergene-Gozukara, 2003; Cavas *et al*., 2005; Muranli and Guner, 2011; Talapatra and Banerjee, 2007) [28, 18, 30, 59, 88, 95].

Ayllon and Garcia-Vazquez, (2000) [18] compared the sensitivity of two fish species European minnow (*Phoxinus phoxinus*) and Mollei (*Poecilia latipinna*) using the micronucleus assay. Cyclophosphamide (40 mg/kg) or mitomycin C (20 mg/kg) was injected to fish intraperitoneally for 24 hours. Various nuclear abnormalities were observed along with micronuclei. A further comparison of sensitivity between 3 fish species *Tilapia rendalli*, *O. niloticus* and *C. carpio* by using the micronucleus assay was done by Grisolia and Starling, (2001) [39]. Waste water from two municipal treatment plants that empty into Lake Paranoa were tested for micronucleus induction. *T. rendalli* was found to be the most sensitive species and *C. carpio* as the most resistant one. Comparisons between the micronuclei induction in fish and mouse was done after treatment with cyclophosphamide, mitomycin C along with other pesticides (Decis 25 CE and kelthane 480 CE). Fish, *T. rendalli* was used and results indicated that both cyclophosphamide and mitomycin C induced MN in both the test organisms. Decis 25 CE and Kelthane 480 CE increased MN frequency in *T. rendalli* at doses 1.0 and 5.0 mg/kg (Grisolia, 2002) [40].

Textile mill effluent was tested by Cavas and Ergene-Gozukara, (2003) [28] for its cytogenotoxic effects on a fish *O. niloticus* by using micronuclei test and analyzing nucleolar organizer region. Dose dependent increase in the frequencies of micronucleated and other NA in erythrocytes were observed while interphase AgNOR frequency in fin cells decreased after 90 and 180 minutes of exposure of textile mill effluent. Cavas and Ergene-Gozukara, (2005a) [30] detected the genotoxic effects of effluents from petroleum refinery and a chromium processing plant. Micronucleus analysis was carried out in gill epithelial cells and peripheral blood erythrocytes and the study showed that both the effluents had genotoxic potential. However, petroleum refinery effluent induced high levels of genetic damage than of chromium processing plant effluent. Koca *et al*. (2005) [49] studied water quality and distribution of some heavy metals in three different organs of *Lipomis gibbosus* from Cine stream (Turkey). Micronucleus formation was studied in erythrocytes from peripheral blood and higher MN frequency in fish caught from the Cine stream was found as compared to control. Zn was found as most accumulated metal in tissue as well as water. In gill tissue primary and secondary gill lamellae got degenerated. Peripheral blood and cephalic kidney of Turbot (*S. maximus*) and Atlantic cod (*G. morua*) were used for testing the genotoxicity caused by crude oil using the micronucleus assay (Barˇsien˙e *et al*., 2006) [21]. Blood cells and kidney cells were found to have significantly higher micronucleated cells when treated with 30ppb of nonylphenol, 0.5 ppm of oil along with alkylphenol and PAH. Group treated with nonylphenol showed fragmented apoptotic cells and significant intertissue differences. MN and nuclear abnormalities in gill and kidney erythrocytes of fish *L. bata* grown in sewage fed fish farm of east Calcutta wetlands was tested by Talapatra and Banerjee, (2007) [88]. Kidney tissue showed an increased value of MN as compared to gill without any statistical difference, but both the tissues showed statistical higher MN and NA as compared to control. Arslan *et al*. (2010) [14] detected micronucleus frequency in aquatic organisms for monitoring pollution of Izmui Bay (Western Turkey), on erythrocytes and gills of mussels (*Mytilus galloprovinciali*) and highest frequency of BN and MN was found in the gill tissue, suggesting the gill tissue as a better marker in micronuclei test. Ayoola *et al*. (2012) [15] investigated the genotoxicity of textile effluent discharge, using micronuclei assay. It was found that textile effluent increased the cytogenetic damage and the chemicals present in the effluent were found to be bioaccumulated and biomagnified in aquatic organisms hence affecting man. A case study in the Abu-rawash area (Egypt) was performed by Lasheen *et al*. (2012) [52]. The toxicity of wastewater effluent on African catfish (*C. garipinus* and *O. niloticus*) was carried out. Along with this, heavy metals (lead, Zinc, copper and cadmium) were also estimated in some tissues of these fishes. Lead and Cadmium metal concentrations were found to be above the permissible level and micronucleus test showed the genotoxic effect of waste water effluent on fish. Kaur *et al*. (2013) [47] evaluated the genotoxicity of dyeing industry effluent in *C. mrigala* by using micronucleus assay. Three sublethal concentrations 24.48%, 12.24% and 6.12% were used for 96 hours of exposure. Blood cells from kidney were used and the maximum effect was observed at 24.48% concentration after 96 hours of exposure. Melo *et al*. (2013) [56] found the usefulness of the micronucleus assay for genotoxic biomonitoring and compared the frequencies of micronucleated erythrocytes and nuclear abnormalities among different species of the electric fish of order Gymntifomes collected from localities of Eastern Amazon. Only one sample collected from the impacted site in the River Caripetuba showed a significant number of NAs which was due to waste water from neighboring mining industries and by burnt fuel released by the local community.

Omar *et al*. (2012) [68] found that high concentrations of heavy metals have a potential genotoxic effects and genotoxicity is possibly related to agricultural and domestic activities. The two species of fish were used *O. nilotics* and *Mugil cephalus*. Both the species collected from the polluted area getting agricultural sewage and domestic non treated discharge and showed a significantly higher MN and NA frequencies as compared to control. Galindo *et al*. (2014) [37] tested genotoxicity of metals in fish *Bathygobius soporato* using micronucleus assay. Erythrocytes were used for testing and samples for testing were taken from tide pools of Salvador city of Brazil. Elevated level of micronucleated cells were observed in the erythrocytes as compared to the control.

Evaluation of the genotoxicity due to heavy metals on the three fish species, i.e. Common carp (*C. carpio*), Prussian carp (*Carassius gibelio*) and Peppered cory (*Corydoras paleatus*) was done. Fish were given 21 days exposure to cadmium (0.005-0.1 mg/l) and copper (0.01-0.25mg/l). Induction of micronuclei and binucleated cells were evaluated in erythrocytes, gill epithelial cells and liver cells. The results suggested the gill and liver to be more sensitive sites as compare to the erythrocytes (Cavas *et al*., 2005) [30]. Yadav and Trivedi, (2009) [99] evaluated the toxicity of three heavy metals, present in Agro-industrial effluent *viz* arsenic,

mercury and copper using micronuclei assay in fish *C. punctatus*. Fish were exposed to sublethal concentrations, i.e. 10% of 96 hours LC_{50} of heavy metal compounds $HgCl_2$ (0.081mg/l), As_2O_3 (6.936mg/l) and $CuSO_4.5H_2O$ (0.407mg/l) for 24, 48, 72, 96 and 168 hours. A significant increase in MN frequency in fish exposed to metal compound was observed and the highest effect of As followed by Hg and least of Cu has been ascertained. Similarly Nagarani *et al*. (2009) [60] assessed the genotoxicity of mercuric chloride in marine fish *Therapon jarbua*. Fish were exposed for 96 hours and significant increase in MN frequency was observed. Highest effect was seen after treatment with 0.25ppm of mercuric chloride. Choudhary *et al*. (2012) [31] detected the genotoxic potential of lead nitrate on fish, *C. punctatus* by assessing the mitotic index in kidney cells and micronucleated erythrocytes in the peripheral blood. The mitotic index decreased with increase in concentration, whereas micronucleated erythrocytes increased with an increase in the concentration and duration of exposure. Talapatra *et al*. (2014) [89] tested three concentrations of zinc (5, 10 and 30 ppm) on fish *H. fossilis* for micronucleus induction. Treatment was given for 24, 48, 72 and 96 hours and time and dose dependent increase in MN induction was observed.

Spleen and peripheral blood erythrocytes of fish *Fathered minnow* were tested for micronuclei induction in response to mitomycin c and cyclophosphamide. Maximum tolerant dose by injecting mitomycin c and cyclophosphamide was found to be 10 and 400 mg/kg respectively. Induction of micronucleus was studied after 1, 2, 4, 8 and 14 days. Significant induction of micronuclei was observed in erythrocytes from spleen, but not from peripheral blood after 8 and 14 days in mitomycin exposed groups. But 400 mg/kg cyclophosphamide failed to significantly induce micronuclei in erythrocytes from any tissue (Winter *et al*., 2007) [97]. Ali *et al*. (2008) [7, 9] reported the gill cells to be more sensitive as compared to kidney tissue and lymphocytes. Fish, *C. punctatus* was exposed to monocrotophos, commonly known as azodrin, one of the organo phosphate pesticides. Gill showed the highest induction of micronuclei. Similarly Sharma and Chadha, (2016) [83] also found gill as most sensitive tissue when tested blood cells from peripheral circulation and blood cells from gill and kidney tissue in fish *C. punctatus* when treated with 4-nonylphenol.

Abdul-Farah *et al*. (2003) [3] had evaluated the genotoxic effects of pentachlorophenol (PCP) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) on fish *C. punctatus*. Sublethal treatment of both the chemicals was given for 96 hours of exposure. Time and dose dependent increase in MN frequency was found in both the groups. PCP was found to be more genotoxic as compared to 2, 4-D in term of MN induction in fish *C. punctatus*. Deltamethrin, class of pyrethroid insecticide significantly induced MN and NAs accompanied by increased lipid peroxidation when fish, *C. punctatus* was exposed to three concentrations (0.4, 0.8 and 1.2µg/l) for 48 and 72 hours (Ansari *et al*., 2009) [13]. Ansari *et al*. (2011) [11] evaluated the cytogenetic and oxidative stress caused by cypermethrin in fish *C. punctatus*. Chromosomal aberration (CA) and micronucleus (MN) tests were used to study the cytogenetic effects. CA and MN were found to show an increase in concentration dependent manner. Similarly, increased oxidative stress was also observed Sharma *et al*., 2021. Srivastava and Singh, (2015) [85] used micronuclei as biomarker for testing genotoxicity of fungicide propiconazole (1.11 and 2.23 mg/l) in fish *C. batrachus*. Peripheral blood was used for micronucleus and binucleus induction and significantly higher values were observed in all the treatments as compared to control (Sharma *et al*., 2020).

Polychromatic erythrocyte frequency

Erythropoeitic toxicity evaluation is an important component for testing the safety level of environmental chemicals, and for erythropoiesis monitoring most popular and convenient method is PCEs count. Fish tends to maintain constant concentration of RBCs under normal conditions. The continuous entry of the new RBCs in the circulation and the destruction of old erythrocytes at the same rate occur, maintaining the dynamic equilibrium between erythropoiesis and destruction of RBCs (Randalli and Farrell, 1992) [75]. The toxicity of a substance can be assessed by PCE/NCE ratio because the toxicant can affect the division and maturation of erythropoietic cells. Decrease in the proportion of PCE/NCE is considered as indicator of mutagen-induced cytotoxicity (Suzuki *et al*., 1989) [87]. The decrease could be due to either direct cytotoxicity or heavy DNA damage leading to cell death or apoptosis. The cytotoxic effect may be ascribable by the direct effect of chemical which may lead to chromosomal breakage as well as destruction of genetic material. These changes in cell may lead to cell death which may result in a reduction of PCE (Suzuki *et al*., 1989) [87]. Along with MN and NA, the ratio of polychromatic erythrocytes to normochromatic erythrocytes is also used for determining the cytotoxicity (Muranli and Guner, 2011) [59]. PCEs count in peripheral blood is one of the promising and convenient methods. The ratio between the two kinds of red blood cells i.e. polychromatic and normochromatic erythrocytes is a useful index to assess cytotoxicity (Udroiu, 2006) [93].

Meier *et al*. (2002) [55] assessed the genetic damage in fish using micronucleus (% polychromatic erythrocytes and MN) and comet assay in laboratory, watersheds and mesocosm. In laboratory 5 model genotoxic agents were tested on bluegill sunfish while in mesocosm caged common carp was exposed for 60 days to alachlor, atrazine, or benzo (a) pyrene. Two watersheds were selected one dominated with urban waste and other with agricultural wastes. All the tested groups showed the significant variation as compared to control. Pacheco and Santos, (2002) [71] exposed fish *Angullia angullia* with benzopyrene and dehyroabietic acid for 180 days. Immature erythrocyte (IE) frequency was determined. A significant decrease in IE frequency was noticed after 30 days of exposure with benzopyrene and after 180 days when treated with dehydroabietic acid. Cavas, (2008) [27] revealed the genotoxicity and cytotoxicity of mercury chloride (MC) and lead acetate (LA). 1 µg/, 5 µg/L and 10 µg/L concentrations of MC and 10 µg/L, 50 µg/L and 100 µg/L concentrations of LA were used. MN frequency was detected in gill tissue, fin epithelial cells and erythrocytes. Along with this PCE/NCE was also estimated in peripheral blood. Gill showed the highest sensitivity having highest MN frequency. While PCE/NCE ratio was found to decrease as compared to control after both the treatments, showing genotoxic as well as the cytotoxic effect of MC and LA. Muranli and Guner, (2011) [59] studied the induction of micronuclei and nuclear abnormalities in Mosquito fish (*Gambusia affinis*) using erythrocytes. Three sublethal concentrations of Lambda-Cyhalothrin (LCT) tested were 1×10^{-4}µg/l, 2×10^{-4}µg/l and 4×10^{-4}µg/l for a period of 6, 12, 24 and 48h. MN, NA and ratio of PCE/NCE was found to decrease after 24 and 48 hour treatment, indicating genotoxic and cytotoxic effect of

lambda-cyhalothrin. Jerbi *et al*. (2011) [46] conducted an experiment to test the genotoxic and cytotoxic effect of formaldehyde and oxytetracycline. Micronucleus assay and PCE/NCE were used for testing genocytotoxicity. Both the tested compounds showed genotoxicity and cytotoxicity, which increased in a time dependent manner. Pesticide pollution has also been studied using the micronucleus assay by Candioti *et al*. (2010) [26]. Evaluation of the genotoxicity and cytotoxicity of the pirimicarb carbanate (insecticide) on *Cnesterodon decemmaculatus* was done after acute exposure. Treatment with 50-100 mg/L concentrations showed an increase in MN frequency. Circulating erythrocytes decreased in proportion and erythrocytes get increased which indicated cellular cytotoxicity. Cavas and Ergene-Gozukara, (2005b) [30]. found metronidazole (antibiotic-antiparasitic) to be genotoxic and cytotoxic to fish of *O. niloticus*. Fish were exposed to 5, 10, 15 mg/L concentrations for 24, 48, 72 hours. Micronucleated polychromatic and micronucleated normochromatic erythrocytes were counted and ratio of PCE/NCE was also calculated. Time and dose dependent increase in micronucleated cells were observed while the ratio of PCE and NCE was found to decrease. Sharma *et al.*, 2014 [84] observed decrease in PCE frequency in *C. punctatus* after acute and sub chronic exposure to 4-nonylphenol.

Comet assay

Since the introduction of the alkaline (pH- 13) Comet assay in 1988, the breadth of applications and the number of investigators using this technique have increased almost exponentially. Olive and Banath, (2006) [67] gave a protocol for comet assay, which could be completed in 24 hours and damage in individual eukaryotic cells could be determined. A large number of studies have been reported that comet assay is more sensitive as compared to the other genotoxic biomarkers (Frenzilli *et al*., 2004) [36]. The simplicity and sensitivity of comet assay made it an adequate test system for biomonitoring of even chronic level exposure (Belpaeme *et al*., 1996) [20]. Mitchelmore and Chipman, (1998) [57] tested the sensitivity of comet assay for environmental monitoring. Trout fish has been used for the study and effect of benzopyrene was tested using four parameters (tail length, tail intensity, tail moment and percent head DNA) and it was concluded that the comet assay is a rapid, easy and sensitive technique. Sunjog *et al*. (2012) [82] tested blood, gill and liver tissue to test DNA damage using the comet assay. Fish samples were taken from Zlatar reservoir as control site and Pestan and Bljanica rivers as polluted sites at Kolubara basin. They suggested tail moment as the most relevant measure for DNA damage. Dose dependent responses were observed in all the tissues. Different workers have used different tissues for the genotoxicity assessment of different chemicals by comet assay. Andrade *et al*. (2004) [11] detected time and dose dependent increase in DNA damage in the erythrocytes of fish *Mugil sp*. after exposure to methyl methanesulphonate (MMS).

Ateeq *et al*. (2005) [15] revealed the genotoxic effect of 2, 4-dichlorophenoxyacetic acid and butachlor on erythrocytes of *C. batrachus* by using the comet assay. Tail length was used as a measure of DNA damage and highest damage at highest concentration and highest duration of exposure was observed. Woo *et al*. (2006) [98] tested the genotoxicity in blood cells of *Paralichthys olivaceus* after exposure to polycyclic aromatic hydrocarbon. The degree of damage was found to increase with an increase in the concentration of PAH and highest damage was found at the station where PAH concentration was found to be maximum. Simoniello *et al*. (2009) [79] used 0.300, 0.150, 0.075 and 0.000 mg/L concentrations of cypermethrin and tested a neotropical fish *Prochilodus lineatus* for genotoxicity using the comet assay. The significantly higher DNA damage was observed after treatment with all the concentrations. Pandey *et al*. (2011) [73] detected the genotoxic effect of profenofos on fish *C. punctatus* using single cell gel electrophoresis. Gill tissue showed a concentration dependent increase in the DNA damage when exposed with sublethal concentrations (0.58ppb, 1.16 ppb and 1.47ppb) for a period of 24, 48, 72 and 96 hours. Guilherme *et al*. (2012) [42] assessed the differential genotoxicity of herbicide Roundup® and its constituent's in fish *A. anguilla.* The fish was treated to Roundup® (58, 116 µgL $^{-1}$) glycophosate (17.9, 35.7 µgL $^{-1}$) and polyethoxylated amine (9.3, 18.6 µgL^{-1}). Blood cells were used for comet analysis and it was observed that both components contributed to the genotoxicity of the herbicide. Nwani *et al*. (2013) [65] assessed the genotoxic effect and oxidative stress in *C. punctatus* after exposure with glyphosate based herbicide. Three sub lethal concentrations 3.25 mg/l, 4.07 mg/l and 6.51 mg/l were used for 1, 7, 14, 21, 28 and 35 days for genotoxicity testing using the comet assay. Highest genotoxicity was observed at 14th day followed by a gradual decline. Nan *et al*. (2013) [63] assessed the genotoxicity and oxidative stress induced by 1-methyl-3-octylimidazolium chloride in fish *Misgurnus anguilliicadatus* using liver cells. It was found that acute toxicity of 1-methyl-3-octylimidazolium chloride above 20 mg/l induced significant induction of genotoxictiy as shown by the increased value of tail length and tail moment in concentration and time dependent manner. Ismail *et al*. (2014) [44] determined the genotoxicity in fish *L. rohita* after exposure to chlorpyrifos. Gill and blood cells were tested and time and dose dependent increase in the DNA damage was found. Gill was suggested as sensitive tissue as compared to blood cells. Javed *et al*., (2016) [45] found that thermal power plant effluent leads to concomitant damage to DNA in gill and liver of fish *C. punctatus*. Significantly higher mean tail length was observed in exposed group compered to fish in the reference group.

Ternjej *et al*. (2010) [92] tested the impact of aluminum (Al) contamination on DNA integrity in erythrocytes of Mosquito fish *Gambusia holbrooki*. This fish inhabited Lake Njivice (Island of Krk, Croatia) and a comparison was done with the same fish inhabiting the unpolluted Lake Ponikve. Three parameters tail length, tail intensity and tail moment were studied. Fish from Lake Njivice showed loss of genome integrity as compared to the fish from Lake Ponikve. Osterauer *et al*. (2011) [70] surveyed the continual entering of platinum into the aquatic environment by road runoff and hospital sewage and raised the concern about its toxicity to organisms. Genotoxicity of platinum was tested at 0, 0.1, 1, 10, 50, 100 and 200 µg/l in Zebrafish (*D. rerio*) and Ramshore snail (*Marisa cornuarietis*) using the single gel electrophoresis. The elevated level of DNA damage was observed in *M. cornuarietis* at 1µg/l and beyond. Ahmed *et al*. (2011) [4] found highest damage in liver tissue followed by kidney and gill tissue when fresh water fish Climbing perch *Anabas testudinus* was tested with lead chloride. Ahmed *et al*. (2012) [5] measured the genotoxic potential of Cadmium in different tissues of fresh water, Climbing perch (*A. testudinus*) using the comet assay. Highest damage was found at highest

concentration. Among different tissues gill showed highest sensitivity. Nagarani *et al*. (2012) [61] identified DNA damage in marine fish *T. jarbua* by comet assay technique when exposed to mercuric chloride ($HgCl_2$). DNA damage was studied in gill, kidney and blood tissue and the gill cells were found to be more sensitive to heavy metal exposure than kidney and blood cells. Abdel-Gawad *et al*. (2011) [1] evaluated DNA damage in fish and aquatic insects in the River Nile. Tail moment was used as a parameter for the assessment of DNA damage and high damage due to the pollutants present in the River Nile was observed. Further high damage was observed in the area from mixed point of agricultural drainage and waste water.

Micronucleus assay in combination to comet assay

One of the advantages of comet and MN assays is that both can be used for the simultaneous assessment of DNA damage in many tissues from the same animal, the comparison of their responses under identical treatment conditions. Thus, these techniques can be used in combination for screening genotoxic effect of chemicals and for investigating the implications of DNA damage and its recovery in the sentinel fish species.Micronucleus test and comet assay are found to be easy, sensitive, rapid and extensively used methods for mutagenicity and genotoxicity testing in various laboratory and field studies (Ali *et al*., 2009; Ateeq *et al*., 2002, 2005; Cavas and Ergene-Gzukara *et al*., 2005a; Jha, 2004; Pandey *et al*., 2006; Sharma *et al*., 2007; Talapatra *et al*., 2006; Nagpure *et al*., 2007; Nwani *et al*., 2010; Ventura Campo de *et al*., 2008;) Buschini *et al*. (2004) [16, 96, 30, 24, 62, 73, 81, 96]. evaluated the mutagenic effect of disinfectant added to the lake water for potabilization in fish *C. carpio*. Comet assay and micronucleus assay were performed on the circulating erythrocytes. Sodium hypochlorite, peracetic acid and chloride dioxide were the three disinfectants used in treating water continuously. Blood samples were taken before adding disinfectant, 3 hours and 10 or 20 days afterward. Sodium hypochlorite and chloride dioxide were the two disinfectants showing the genotoxicity. Russo *et al*. (2004) [77] selected two natural environments for analyzing the biological damage (polluted water of Sarno River and Astroni natural reserve as negative control). *Gambusia holbrooki* was used as test organism and micronuclei (MN) test and single cell gel electrophoresis (the Comet assay) were used for environmental stress evaluation. Both the parameters showed high genotoxicity in the samples from the Sarno River as compared with Astroni. Matsumoto *et al*. (2006) [54] used comet and micronucleus assay in erythrocytes of *O. niloticus. S*tudied samples of water were taken from Catfish Brook in Franca, a city in the Brazilian state of S˜ao Paulo. The highest DNA damage was observed in the samples from the tanning region containing chromium effluent. Comet assay revealed the maximum damage from chromium containing tannery effluent site. Ali *et al*. (2009) found the genotoxicity of chlorpyrifos in fish *C. punctatus*. Lymphocyte and gill cells were used for the testing. Highest induction of MN was observed at 14th day while highest DNA damage was observed at 5th day. Kumar *et al*. (2010) [51] investigated genotoxicity of malathion (pesticide) in kidney, gill and lymphocytes of *C. punctatus* using the micronucleus test and comet assay. A concentration dependent increase in DNA damage was observed up to three days followed by a nonlinear decrease with the duration of exposure. Comparison of DNA damage occurred among various tissues showed the sensitivity of gill tissue to malathion. Nwani *et al*. (2010) tested the mutagenic effect of carbosulfan (insecticide) in fish *C. punctatus*. Both the parameters (MN and comet assay) were used for testing. Fish were exposed to three sublethal concentrations of carbosulfan (67, 134 and 201µg/l) for 96 hours. Highest damage was observed at 96 hours of exposure in erythrocytes and gill cells. Diekmann *et al*. (2004) [33] examined the genotoxic potential of 4 nitrous oxide in a complete life cycle of Zebrafish (*D. rerio*) by using the comet assay and micronucleus assay. Lower reproductivity and increased genotoxicity were observed. Ferraro *et al*. (2004) [35] assessed the mutagenic effect of 2 months exposure to tributylin (TBT) and lead (Pb) on *Hoplias malabaricus.* Comet assay, micronucleus assay and chromosomal aberration were used as genetic biomarkers. Blood cells were used for comet assay and results indicated genotoxicity with Pb but the results were not positive with TBT. On the other hand the micronucleus and chromosomal assay indicated the mutagenic effect of both TBT and PB. Caliani *et al*. (2009) [25] assessed the potential genotoxicity of produced water (PW) containing residual hydrocarbon, trace elements, naturally occurring radioactive material and potential toxic treatment chemicals such as biocides, dispersants, detergents and scale inhibitors used in oil production to Mosquito fish (*G. affinis*) for 30 days. Genotoxicity was evaluated by the comet assay and micronucleus test. A positive correlation between MN and PAH metabolites was found. Nwani *et al*. (2011) [65] investigated the potential hazard associated with atrazine based herbicide on fish *C. punctatus* using the micronucleus assay and single cell gel electrophoresis. Gill tissue and erythrocytes were used for the analysis. Three sublethal concentrations 8.48, 5.30 and 4.24 mg/l for a period of 1, 3, 5, 7, 14, 21, 28 and 35 days were used for the exposure. Highest MN frequency in blood cells was on the 7th day with the highest concentration, while longest tail was observed on 5th day followed by non-linear decline. Rocco *et al*. (2012) [76] assessed the genotoxic effects of erythromycin and lincomycin and combination of these two antibiotics on the genome of zebra fish by using micronucleus assay on erythrocytes and the comet assay on erythrocytes and hepatocytes. It was found that treatment led to increase in DNA migration (tail moment) and significant increase in micronucleus frequency. Repair mechanism was also analyzed by removing genotoxic agent and it was found that only a few cells displaced the damaged cells. Faßbender and Braunbeck, (2013) [34] performed a multigenerational study to assess the reproductive and the genotoxic effect of methyl methanesulfonate in Zebrafish after chronic exposure. Genotoxicity testing was done using comet and micronucleus assay. The significant genotoxic effect was observed in liver, gills and gonads while no detrimental effect on growth was seen. Palanikumar *et al*. (2013) [72] found significant induction of MN and BN with the increasing concentration of naphthalene when compared to control and solvent control group. Maximum nuclear abnormality frequency was recorded for 1.24 µg/l of naphthalene. The DNA damage index was measured as percent comet tail in the gill and liver tissues of control and treatment groups. DNA damage increased with increase in concentration. Kumar *et al*. (2013) [50] conducted an experiment to assess the genotoxicity and oxidative stress of potassium dichromate in fish *C. carpio*. Genotoxicity was assessed in blood and gill tissue using comet and micronucleus assay and oxidative stress (OS) was estimated in liver, kidney and gill tissues. Both the tissues

showed a concentration dependent increase in MN frequency and comet tail length. Tan *et al*. (2013) [95] observed a significant induction of micronuclei and other nuclear abnormalities as well as olive tail moment and percent tail DNA in fish *C. auratus* after exposure with acrylamide. Mutagenic effect to 5, 10 and 20 mg/l acrylamide for a period of 96 hours was assessed using peripheral blood. Ali *et al*. (2014) [8] used *C. punctatus* for study of genotoxicity and oxidative stress response caused by dimethoate (organophosphate insecticide). Concentration and time dependent increase in MN induction and DNA damage were found. 3 week exposure of dialkyl phthalate, bisphenol A, tetrabromodiphenyl ether (50 ppb of each) and nonylphenol (30ppb) as well as the mixture were given to fish *S. maximus*. Highest effect was observed in a mixture of sea oil and alkylated phenols. Bisphenol and tetrabromodiphenylether also showed the significant induction of MN and NA (Bolognesi *et al*., 2006) [22].

Cavalcante *et al*. (2008) [27] who tested blood and gill cells of fish and found high damage at 6 hours of exposure, but at 24 hours blood cells showed decreased damage and in contrast to this gill showed high damage at 24 hours. Similarly Banu *et al.* (2001) [19] showed that monocrotophos treatment in fish, *Tilapia mossambica* lead to time dependent reduction in DNA damage. 24 and 48 hours of exposure show highest damage followed by a drop in the value up to 96 hours of exposure at all the concentrations and value almost return to the control value. Further Gulsoy *et al.* (2015) [43] reported that when Zebra fish (*D. rerio*) were exposed to borax, the highest DNA damage was observed at 24 hours, followed by decrease at 48 and 72 hours and again increase in the value was observed at 96 hours, while treatment with boric acid induced highest effect at 96 hours of exposure. Ali and Kumar, (2008) [7, 9] reported that when fish *C. punctatus* were exposed to monocrotophos gill, liver and lymphocytes show highest damage at 4th day followed by decline in value afterwards. Mohanty *et al*. (2011) [58] found the highest damage in liver of fish *L. rohita* after treatment with organophosphate pesticide shows highest damage at 72 hours and then DNA damage get decreased at 96 hours when treated with. Ahmed *et al.* (2011) [4] reported significant induction of DNA damage at 48 and 96 hours of exposure followed by a decline after 192 hours of exposure.

Gill cells were found to be more sensitive than lymphocytes, blood erythrocytes, kidney or liver cells by various researchers (Ateeq *et al.*, 2005; Ali and Kumar, 2008; Ali *et al*., 2008, 2009; Nwani *et al.*, 2010; Pandey *et al*., 2006; Sharma *et al*., 2007). On the other hand Akter *et al*. (2009) [16, 6, 7, 9, 64, 73, 81] reported high apoptotic cells in liver tissue of *A. testudineus* when exposed to heavy metals. Likewise Kilemade *et al*. (2004) [48] found a sensitivity of liver tissue over gill, blood and spleen in Juvenile turbot. The liver may also show high genotoxicity as lower molecular sulphate conjugates could favor their removal by kidney and gill (Vazquez-Duhalt *et al*., 2005) [95]. Uguz *et al*. (2006) [94] found time dependent accumulation of 4-NP in liver cells of fish *Onchirynchus mykiss*.

These studies suggested the usefulness of DNA damage assessment using comet assay in combination with micronucleus test to investigate the possible mechanism of genotoxicity in fish.

Assessing DNA repair activity

Another aspect studied in this review is paucity of knowledge concerning DNA repair capabilities. To enrich this scientific field such studies can be considered challenging. The knowledge of DNA repair in the tested species is important as it represents the first line of defense against genotoxicants. Guilherme *et al*. (2014) [41] studied the genotoxic effect of Round up® and Garlon® in European eel (*A. anguilla*) and the extent of recovery was also studied after cessation of exposure and blood cells were found to be recovered more successfully. Marques *et al.* (2014) [53] evaluated the mechanism of genotoxicity induced by glyphosate based herbicide and repair in *A. anguilla* upon exposure and post exposure period. Exposure was given for 3 days and post exposure period was 1-14 days. It was found that the DNA integrity returned to control level after 1 day post exposure. Damage dropped to control value suggested the possibility of complete turnover of fish erythrocytes and other cells. The life span of erythrocytes in fish is from 1-3 months (Udroiu, 2006) [93]. Similar results were found by Bony *et al*., (2008) [23] in Trout in response to vineyard pesticide, in Carp and Bullheads (Pandrangi *et al*., 1995) [74] and in Chub (Devaux *et al*., 1998) [32]. Similar results were also found by Mohanty *et al.* (2011) [58] in fish *L. rohita* which were exposed to organophosate pesticide. They observed the DNA break reduction in liver tissue after 3 hours and the fish returned to control levels damage in 24 hours. This may be due to DNA repair system which may lead to elimination of DNA lesions. Abdel-Tawwab, (2012) [2] observed that fish gradually recover close to the control values after 3 to 4 weeks of recovery in the Nile tilapia after exposure to commercial petroleum fuels. Sharma and Chadha, (2017) [82] observed significant reduction in DNA damage after 30 days recovery period when fish *C. punctatus* exposed with 4-nonylphenol for 90 days sub chronic exposure.

Conclusion

As evident from the review the toxicological relevance of micronucleus assay, polychromatic erythrocyte frequency and comet assay is well defined as the parameters are simple to score, accurate and applicable to different cells. MN and comet assay can be used to identify potential biomarkers that allow the identification of early genotoxic damage in a simple and effective manner and proves to be effective tools for genotoxicity evaluation. Finally the studies dealing with repair of DNA damage can be used to illustrate the repair and cellular turnover.

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