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Abstract

The application of fish erythrocyte micronucleus assay in pollution monitoring- particularly in water has been described as sensitive biodetector of genotoxicants. The pervasiveness of this technique has been characterized by varieties of sensitivities and adequacies in forms of early warning of eco-damage and toxicity, stresses on the health of organisms and ecosystems. Since biodiversity-rich freshwater ecosystems are currently declining faster than the marine or land ecosystems, making them the world’s most vulnerable habitats and their sustainability being threatened by anthropocentrism, fish micronucleus test holds great promise for continuous and effective pollution evaluation. This paper briefly explores the biomonitoring potentials of micronucleus test on waterborne pollutants. It then considers the interplay between such micronucleus formations and certain widespread environmental pollutants. While acknowledging the fact that both peripheral and kidney bloods are sensitive to the genetic damage induced by the aquatic toxicants, the paper concludes that bioindicators offer several types of unique information not available from other methods. The sampling of peripheral blood is appropriate and sufficient for biomonitoring projects, as it allows collecting several samples from the same individual, without having to sacrifice it.

**Key words:** Genotoxicity, aquatic ecosystem, genetic marker, dose-time effect
Introduction

Many toxic and potentially toxic chemical substances, some of natural origin and others due to human activities, are released into the environment daily. It is difficult to practice even elementary hygiene without sufficient quantities of water free of these contaminants (UNFPA, 2001). In addition, it is necessary to protect the water sources themselves from faecal contamination and agricultural and industrial pollutants. In developing countries, 90 to 95 percent of all sewage and 70 percent of all industrial wastes are dumped untreated into surface water (UNFPA, 2001). Due to the increasing environmental exposure to these agents, the need for biomonitoring terrestrial and aquatic ecosystems, especially in regions compromised by chemical pollution is paramount (Silva et al., 2003; Matsumoto et al., 2003 and 2005; Avishai et al., 2002; Mitchelmore and Chipman, 1998).

Genotoxic pollution of aquatic ecosystem describes the introduction of contaminants with mutagenic, teratogenic and/or carcinogenic potentials into its principal media and genome of the resident organisms (Environ Health Perspect, 1996; Fagre et al., 2008; Badr and El-Dib, 1978). Genotoxicity is a deleterious action, which affects a cell’s genetic material affecting its integrity (WHO, 1997; Environ Health Perspect, 1996). Several genotoxic substances are known to be mutagenic and carcinogenic, specifically those capable of causing genetic mutation and of contributing to the development of human tumors or cancers (Hayashi et al., 1998; Fagre et al., 2008; Shugart, 1988; Black et al., 1983; Hose, 1985; Baumann and Mac, 1988; Hose et al., 1984). These include certain chemical compounds like heavy metals (Matsumoto et al., 2005; Igwilo et al., 2006; Matsumoto, 2003; Pruski and Dixon, 2002; Lee and Steinert, 2003) microbial toxins (Environ Health Perspect, 1996) and polycyclic aromatic hydrocarbons (PAHs) (IARC, 1983; Santodonato et al., 1981; Black et al., 1983; Germain et al., 1993). These genotoxicants have been reported to cause mutations because they form strong covalent bonds with DNA, resulting in the formation of DNA adducts preventing accurate replication (Hartwell et al., 2000; Luch, 2005; Varanasi et al., 1989). Genotoxins affecting germ cells (sperm and egg cells) can pass genetic changes down to descendants (Hartwell et al., 2000) and have been implicated to be against sustainable development principles by WHO (1997; 2002b) portraying them as significant factors in congenital anomalies, which account for 589,000 deaths annually.

Biomarkers are biological responses to environmental chemicals at the individual level or below demonstrating departure from normal status (Walker et al., 2003). Biomarker responses may be at the molecular, cellular or ‘whole organism’ level. An important thing to emphasize about biomarkers is that they represent measurements of effects, which can be related to the presence of particular levels of environmental chemical; they provide a means of interpreting environmental levels of pollutants in biological terms. Biological responses at higher organizational levels- population, community, and ecosystem- are considered as bioindicators (Figure 1). The most widely used classes of biomarkers are biomarkers of exposure and biomarkers of effect. Biomarkers of exposure are those that indicate exposure of the organism to chemicals, but do not give information of the degree of adverse effect that this change causes. Biomarkers of ‘effect’, or more correctly ‘toxic effect’ (because all biomarkers by definition show an effect), are those which demonstrate an adverse effect on the organism (Walker et al., 2003). Fish are excellent subjects for the study of the mutagenic and carcinogenic potential of contaminants present in water. This is so because they can metabolize, concentrate, and store waterborne pollutants (Park et al., 1993; Ali and El-Shehawi, 2007). Since fish often respond to toxicants in a similar way to higher vertebrates with fast responses on low concentrations of direct acting toxicants (Poelé and Strik, 1975; Koeman et al., 1977; Poelé, 1977; Sloof, 1977; Badr and El-Dib, 1978), they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans. The main application for model systems using fish is to determine the distribution and effects of chemical
contaminants in the aquatic environment (Al-Sabti and Metcalfe, 1995).

Fig. 1: Schematic relationship of linkages between responses at different organizational level (Biomarker Strategy Model). Source: Walker et al. (2003).

Micronucleus (MN) assay is an ideal monitoring system that uses aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. Research reports maintained that it can be applicable to freshwater and marine fishes and that gill cells are more sensitive than the hematopoietic cells to micronucleus inducing agents (Hayashi et al., 1998). Micronuclei are cytoplasmic chromatin-containing bodies formed when acentric chromosome fragments or chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division (Palhares and Grisolia, 2002; Fagr et al., 2008). This genetic damage arises as results of chromosome or spindle abnormalities leads to micronucleus formation. Recent research reports maintained that micronucleus formation in freshwater and marine fish is a function of water pollution caused primarily by heavy metals and polycyclic aromatic hydrocarbons. According to Hartwell et al. (2000) and (Fagr et al., 2008), the incidence of micronuclei in fish and other aquatic life serves as an index of these types of damage and counting of micronuclei is much faster and less technically demanding than scoring of chromosomal aberrations. The micronucleus assay has been widely used to screen for chemicals that cause these types of damage (Palhares and Grisolia, 2002; Campana et al., 1999; De flora et al., 1993; Kligerman, 1982).

In genotoxic pollution of freshwater, the toxicants are mostly introduced into the water bodies through anthropogenic activities such as industrial, agricultural, domestic, and urban activities. However, due to ecological-level interactions, the health of the biota that depends on it is adversely compromised through contact with
hazardous chemicals capable of damaging the DNA and perpetuating the irreversible effects evidenced by micronuclei formations. These micronuclei serve as useful marker for environmental biomonitoring of the aquatic chemical contaminants. This damage tends to be irreversible and continues manifesting in future generations through heredity. The species diversity of the impacted ecosystem would be drastically reduced and humans occupying higher trophic levels become threatened through sufficient biomagnification along the food chain. These pollutants have similar genotoxic effect on biological systems as they can induce chromosomal rearrangements and aneuploidy (change in chromosome number).

**Micronucleus as Aquatic Pollution Biomarker**

The first evidence that environmental contaminants might have an influence on the genetic materials of the population came about seven decades ago with the discovery that mutations were induced by high-energy radiation. However, the subsequent development of nuclear energy added a new dimension and enhanced awareness of the problem of genetic hazards. Current awareness of the potential hazards of pollutants in the aquatic environment has stimulated much interest in the use of fish as indicators for monitoring carcinogens, teratogens, clastogens and mutagens. This is because aquatic environment serves as convenient repositories for man’s biological and technological wastes (Cajaraville et al., 2000). Aquatic animals have often been used as assay to evaluate surface water (Brugs et al., 1977, Čarins et al., 1975). Substances displaying mutagenic, teratogenic and carcinogenic potentials are easily evaluated because of high sensitivity of these organisms to these pollutants on low concentrations (Sloof, 1977, Koeman et al., 1977, Poole and strik, 1975). Rodriguez –Cea et al. (2003) determined the sensitivity of micronucleus test in freshwater fish species for application in field surveys. The author studied three fish species namely: Brown trout (*Salmo trutta*), European eel (*Anguilla anguilla*) and European minnow (*Phoxinus phoxinus*) for their use as in situ pollution biomarker by measuring the micronucleus indices of their renal erythrocytes. They used cyclophosphamide, colchicine and cadmium as pollutants to examine their genotoxicity. Cyclophosphamide (CP) is an alkylating agent. It causes alkylation of the purine ring, and as a result, there is miscoding and blockade of DNA replication. Schular et al. (1997) evaluated the centromeric labelling to distinguish micronuclei induced by chromosomal loss and breakage in vitro. The in vitro micronucleus assay has been used to characterize the origin of the micronuclei induced by cyclophosphamide (Fagr et al., 2008). Chorvatovicova and sandula (1995) recommended the use of cyclophosphamide in chromosome aberration tests, sister chromatid exchanges and micronucleus (MN) formation in vitro and in vivo. This drug is mutagenic, usually used as positive controls in in vivo tests of short duration.

Fish serves as useful genetic model for the evaluation of pollution in aquatic ecosystems (Mitchell and Kennedy, 1992, Park et al., 1993). The erythrocyte micronucleus test has been used with different fish species to monitor aquatic pollutants displaying mutagenic features in developed countries (De Flora et al., 1993; Saotome and Hayashi, 2003; Pantaleao et al., 2006). Kligerman (1982) demonstrated that fish inhabiting polluted waters have higher frequencies of micronuclei. The micronuclei frequencies may vary according to the season, the kind of pollution involved, and the species of fish. In laboratory tests involving fish, several substances have been shown to have genotoxic potential (Odeigah and Osaneyinpeju, 1995; Minissi et al., 1996) while others have proven innocuous (Belpaeme et al., 1996).

The micronuclei represent acentic chromosome fragments or whole chromosomes lost during cellular anaphase stage of mitotic cell division (Hartwell et al., 2000; Palhares and Grisolia, 2002). These substances are easy to visualize in erythrocytes and therefore often used as a measure of chromosomal aberration (Rabello-Gay, 1991). Obiakor et al. (2010a) made similar report while working with *Synodontis clarias* and *Tilapia nilotica* from freshwater of the Anambra River. The authors documented the micronuclei rates of these species, validating them as index of cytogenetic damage, monitoring of aquatic genotoxicants and other sublethal concentrations of chemical pollutants. In fish, the kidney is
Genotoxic alterations induced by River Oyi were investigated based on the karyomorphological analysis and micronucleus assay in *Clarias gariepinus* exposed to its water for 10 and 28 days, respectively. A standard control experiment containing groundwater of drinking quality was set up to monitor deviation. Fish exposed to the water had significantly higher (P<0.05) number of chromosomal aberrations and micronuclei compared to the control fish exposed at the specified period. After exposure of the fish to the River Oyi water for 28 days, significant (P<0.05) increase in the genotoxic capacity of the water was evidenced. The study further revealed dose and time response relationship and effect (Obiakor et al., 2010c). Upon fish exposure to toxins, defective erythrocytes undergo passage from the kidney into the peripheral blood, from where they are removed by the hemocatheteris organ (Palhares and Grisolia, 2002). One of the hypotheses of this study was that the examination of kidney erythrocytes would provide more sensitive detection of micronuclei frequencies than peripheral blood erythrocytes under natural conditions in priority chemically polluted freshwater. In fish, the micronucleus test is usually based on erythrocytes, but liver and gill tissues have been used (Al-Sabti and Metcalfe, 1995). In mammals, young bone marrow erythrocyte can be distinguished quite easily from the mature ones by examining the Giemsa staining pattern of the cells. However, in fish, this distinction is not feasible. Rodriguez-Forero (1995) suggested that young fish erythrocytes stain as basophils with Giemsa.

Ahmad et al. (2002) studied the effect of pentachlorophenol on fish genome using haematoxylin-eosin technique and discovered that the frequency of micronucleated erythrocytes increases with increased time of exposure to pentachlorophenol. Computer image analysis of morphological variations of erythrocyte indicated a 1:5 ratio of micronuclei and main nucleus accompanied by a reduction in cell volume by 600 dot units. Campana et al. (1999) studied the genotoxicity of pyrethroid lambdacyhalothrin using micronucleus test in erythrocytes of Cheirodon (Interruptus interruptus). Results obtained demonstrated the genotoxic effects of the pyrethroid in the experimental model employed and maintained that the variation in the micronuclei frequencies in the different sampling times observed could be related to the blood cell kinetics and the erythrocyte replacement. The results could be considered as a validation of the MN test in fish for the assessment of genotoxic pollutants.

Fagr et al. (2008) evaluated micronucleus assay in fish genome as a sensitivie monitor for aquatic pollution using three tilapia species (*Oreochromis niloticus*, *Oreochromis aureus* and *Tilapia zilli*) and *Clarias gariepinus* from four locations (River Nile, drainage at Abou Homos, Kafr Eldawar and Lake Mariout) that represent different levels of contaminants (Ali and El-Shehawi, 2007) in Egypt. Results reveal that the four fish species represent various degrees of sensitivity in monitoring genetic damage (especially clastogenic effect). This is indicated by variations in averages of the micronucleated cells among species at various locations (Table 2.1). As previously mentioned by Ali and El-Shehawi (2007), these locations display differential environmental stress. On the other hand, peripheral blood of *Clarias gariepinus* was shown to be very sensitive in formation of micronucleus depending upon the environmental stress, and might be by other factors (Fagr et al., 2008).

Palhares and Grisolia (2002) compared between the micronuclei frequencies of kidney and gill erythrocytes in tilapia fish, following mitomycin C treatment detecting no significant difference between them. Similarly, Manna and Sadhukan (1986) maintained that there was no statistically significant difference between the frequency of micronuclei in gill and kidney cells after irradiation in the two tissues. A contrasting observation was recorded by Fagr et al. (2008) that the peripheral blood was more sensitive with higher micronuclei frequencies for the damage induced by the aquatic contaminants (approximately 150%) compared with kidney erythrocytes while working on several fish species sourced from different locations in Egypt displaying differential environmental stress. Shukla et al. (2007) and Witeska and kosciuk (2003) suggested that
waterborne heavy metals, initially bound to the gills as they were in direct contact with ambient medium and the main site of water movement (Shukla et al., 2007) and subsequently deposited in other tissues, might affect the fish, even if toxic agent was removed from the water. Similarly, Obiakor (2010) and Obiakor et al. (2010a) evaluated the genotoxic status of the Anambra River employing two preponderant fish species of the river and possibility of congenital disease outbreak among the resident population using the river by micronucleus assay (Figure 2). The following criteria for MN identification were adopted from Fenech et al., (2003); the diameter of the micronucleus (MN) should be less than one-third of the main nucleus; MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary; and MN should have similar staining as the main nucleus. According to the authors, the fish species showed varying degree of micronucleus frequencies in their genome of sampled peripheral (gill) and kidney blood, portraying them to be sensitive with peripheral blood being significantly higher (P<0.05).

**Fig. 2:** Photomicrographs showing micronucleated erythrocytes (MN) from Tilapia nilotica and Synodontis clarias caught from Otuocha (a) and Onono (b) axes of Anambra River, respectively in rainy season. Source: Obiakor (2010) and Obiakor et al. (2010a)

**Micronucleus Formations and Environmental Pollutants**

Several synthetic compounds such as pesticides, metals, drugs, and plastics have become increasingly widespread in our environment. These pollutants have lethal effects on aquatic and even terrestrial organisms. Among them, heavy metals and persistent organic pollutant (POP) such as polycyclic aromatic hydrocarbons (PAHs) have more profound application because of their source and environmental mobility. Various authors have reported on the genotoxicity of these chemical substances and their irreversibility.

There are some studies to show the genotoxicity of heavy metals on aquatic organisms, such as copper (Guecheva et al., 2001; Arkhipchuk and Garanko, 2005) but actual mechanisms of its genotoxicity are poorly discussed (Bagdonas and Vosyiene, 2006). One of the possible paths of copper genotoxicity is induction of oxidative stress and production of DNA damaging reactive oxygen species (Gabbianelli et al., 2003). According to Bagdonas and Vosyiene (2006), little is known about copper (Cu) and zinc (Zn) genotoxicity. These heavy metals have been documented to induce micronuclei formation in aquatic organisms. Guecheva et al. (2001) elucidated the genotoxicity of copper sulphates to planaria by means of Comet assay, discovering elevated levels of DNA strand breakage and inhibition of DNA repair of planaria preexposed to methylmethan sulphonate. This inhibition of DNA repair enzymes according to the authors could be caused by a non-specific binding of Cu$^{2+}$ cations to essential sites in the enzyme molecule. This reflects the formation of micronuclei due to this DNA damage and repair by copper. Similarly, Gabbianelli et al. (2003) had reported genotoxic effect of copper in erythrocytes of Sparus aurata while Bagdonas and Vosyiene (2006) documented the single and joint action toxicity of copper and zinc to Rainbow trout, a freshwater fish. However, Obiakor et al. (2010b), showed the
The genotoxicity of copper, zinc and their binary mixture to two species of fish using micronucleus test (Figure 3). The authors, validating the technique revealed the genotoxic effect of these metallic species at short duration and independent of toxicant concentrations.

Fig. 3: Photomicrograph showing micronucleated erythrocytes (arrows) in Synodontis clarias after treatment with binary mixture of Cu and Zn. Source: Obiakor et al. (2010b).

Similar observations have been made in various mammalian cells. Several studies have assessed the genotoxicity of copper sulphate following oral or parenteral exposure in vivo. Significant increases in the occurrence of micronuclei have been observed in chick bone marrow cells and erythrocytes (Bhuny and Jena, 1996) and mouse bone marrow cells (Bhuny and Pati, 1987). Positive results have been found in studies testing for DNA damage in vitro in multicellular organisms. Errors in DNA synthesis by viral DNA polymerase (Sirover and Loeb, 1976), a reduction in DNA synthesis (Garrett and Lewtas, 1983; Sirover and Loeb, 1976), and an increase in the occurrence of DNA strand breaks (Sideris et al., 1988; Sina et al., 1983) have been observed. The increased in sister chromatid exchange in Chinese hamster cells (Sideris et al., 1988) is consistent with the clastogenic effects observed in in vivo assays. There are also indications of weak clastogenic effects following zinc exposure. A dominant lethal study in mice failed to show a mutagenic potential for zinc. However chromosomal aberrations have been observed in bone marrow cells following in vivo exposure to zinc (Vilkina et al., 1978). This effect was observed in rats exposed to 14.8mg zinc/kg/day as zinc chloride in drinking water (Kowalska-Wochna et al., 1988), mice given intraperitoneal injections of 3.6mg zinc/kg/day as zinc chloride (Gupta et al., 1991), and mice exposed to zinc oxide by inhalation (Voroshilin et al., 1978), chromosomal aberrations caused by zinc were also observed in the bone marrow cell of mice maintained on a low calcium diet (Deknudt and Gerber, 1979). Calcium may be displaced by zinc in calcium-depleted conditions, leading to chromosome breaks and/or interfering in the repair process (Deknudt and Gerber, 1979). In vivo exposure of zinc also resulted in single strand breaks, as measure by the comet assay in mice (Banu et al., 2001). An increased incidence of sister chromatid exchange was observed in bone marrow cells of rats exposed to 17.5mg zinc/kg/day as zinc chloride in drinking water (Kowalska-Wochna et al., 1988) while Bagdonas and Vosyliene (2006) documented significant increase of micronuclei in fish exposed to varying concentrations of zinc sulphate.

Exposure of fish to PAHs has been associated with various teratogenic effects. After a seven-day exposure to naphthalene at a concentration of 239µg/L, teratogenic deformities were seen in 6% of embryos of largemouth bass (Micropterus salmoides) (Black et al., 1983). Deformities were also observed in 43% of rainbow trout (Oncorhynchus mykiss) exposed for 27 days to 85 µg/L of phenanthrene (Black et al., 1983). Eyes anomalies were detected in 7% of rainbow trout alevin exposed for 36 days to 0.2 µg/L of B[a] P and in 17% of those exposed to 0.3 µg/L. The
lowest concentration at which effects were observed include 0.1 µg/L (36-day LOEC for eye anomalies in 2% of rainbow trout fry) (Hose et al., 1984). Genotoxic and neoplastic effects have been reported in both vertebrate and invertebrate organisms following metabolism of certain PAHs. Of the compounds selected for the environmental assessment, such effects were observed under laboratory conditions for B[a] P, phenanthrene, and naphthalene (Shugart, 1988; Black et al., 1983; Hose et al., 1984; Hose, 1985). Exposure of fish to PAHs leads to clastogenic effects resulting from DNA damage. For example, a formation of numerous secondary micronuclei is observed in red cells of rainbow trout embryos exposed for 36 days to 0.1 µg/L of B[a] P (Hose et al., 1984). A 24-week exposure (with two six-hour periods per week) to 150 to 240 µg/L of B[a] P caused hepatic neoplastic tumour in 10% of the guppies tested (Hawkins et al., 1990). In aquatic invertebrates, B[a] P (0.5 µg/L) was shown to be clastogenic (chromosomal aberrations and secondary micronuclei) and teratogenic (deformed gastrula) to eggs of sea urchin (paracentrotus purpuratus) following exposure for 48 hours (Hose, 1985).

**Conclusion**

The aquatic environment makes up the major part of our environment and resources; therefore, its safety is directly related to the safety of our health and food security. Biomarkers and bioindicators using fish micronucleus assay in eco-genotoxicology offers several types of unique information not available from other methods. These include:

- early warning on environmental damage
- the integrated effect of a variety of environmental stresses on the health of an organism and the population, community, and ecosystem
- relationships between the individual responses of exposed organisms to pollution and the effects at the population level
- early warning of potential harm to human health based on the responses of wildlife to population, and

the effectiveness of remediation efforts in decontaminating waterways (Villela et al., 2006).

Genotoxic evaluation of aquatic environment is a key mechanism for translating the principle of sustainable development into action. Genotoxic pollutants have been associated with gene mutation (mutagenic) and proliferation of tissue (carcinogenic potential). These chemicals are capable of transforming the future generations if unchecked because of its potential to cause genetic hazards. Though fish die first, next is human (Okpokwasili, 2009). Sampling of peripheral blood is appropriate and sufficient for biomonitoring projects, as it allows collecting several samples from the same individuals without having to sacrifice it (Lyne et al., 1992). Micronucleus assay has not received considerable attention in environmental biotechnology and management thereby undermining the lethal and sub-lethal effects of certain eco-genotoxicants. These pollutants have been reported to be eco-toxic in developed countries (Germain et al., 1993). However, little or no information exists in Nigerian Rivers. Studies employing these biomarkers would invariably establish the genotoxic concentrations of a given aquatic body and call for proactive measures in control.

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