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Genotoxic Potential of Pesticides in the Peripheral Blood Erythrocytes of Fish *(Oreochromis mossambicus)*

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ABSTRACT

The marine environment as a greater part of the world ecosystem is vital source of nourishment and its safety is linked to our health. The micronucleus (MN) assay has been used to evaluate genotoxicity of many compounds in polluted marine ecosystems. The aim of this study is to verify the efficiency of the micronucleus assay in laboratory, using erythrocytes of the tilapia specie (*Oreochromis mossambicus)* as genotoxicity biomarker. In the present study, the genotoxic potential of pesticides was carried out in the peripheral blood erythrocytes of fish *(Oreochromis mossambicus)* using micronucleus (MN) assay. Different doses of organophosphate pesticides (chlorpyrifos and malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide were injected intraperetonially and specimen were sacrificed after 24 and 48 h. Peripheral blood samples smears were stained with Giemsa, MN frequencies were counted and statistically analyzed. Our results revealed significant dose dependent increase in the frequencies of micronuclei in pesticide treated fish as compare to control. The highest MN frequencies were recorded after 48 h cypermethrin exposure and the lowest MN frequencies were recorded after 48 h buctril exposure. The genotoxicity of pesticides on fish at 48 h exposure in the present study is found to be in the order of cypermethrin, chlorpyrifos, malathion, lambda-cyhalothrin and buctril, in peripheral blood erythrocytes. Result of the present study suggests use of the micronucleus test in fish erythrocyte as a sensitive indicator for evaluation and assessment of the carcinogenic and mutagenic compounds in marine environment.

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Authors' Contribution
NS conceived and designed the study. GZN performed the experiments and analyzed the data. AMA helped in experimental work and data analysis. NS and GZN wrote the article.

Key words
Oreochromis mossambicus, **Chlorpyrifos, Buctril, Malathion, Micronucleus**

INTRODUCTION

Agrochemicals are employed worldwide in agriculture to protect crop from pests, weeds, pathogens and parasites. The pesticides enter the aquatic ecosystem through runoff from agricultural fields that lead to the pollution of aquatic environments such as rivers, ponds, lakes etc. The bioaccumulation and persistence of these pollutants in the aquatic environment pose a serious threat to marine life and to human beings indirectly through the food chain (Binelli and Provini, 2004). The aquatic ecosystem as a greater part of the natural environment is faced with the threat of a shrinking genetic base and biodiversity due to indiscriminate use of pesticides (Omitoyin *et al*., 2006). The majority of these hazardous chemicals are mutagenic in nature (Garaj-Vrhovac and Zeljezic, 2002), either linked to the cancers or might lead to developmental deficits (Leiss and Savitz, 1995; Arbuckle and Server, 1998). Micronucleus (MN) is regarded as the marker of cytogenetic damage, appearing after the impact of genotoxic compound. Micronuclei are small masses of cytoplasmic chromatin outside the main nucleus of cells, which can originate from a chromosome break or spindle abnormalities (Heddle *et al.*, 1991), *i.e.*, there are entire or chromosome fragments that were not incorporated inside the nucleus of the daughter cell during cell division and that appear as a small roundish dark structure, identical in appearance to the cell nucleus (Bombail *et al.*, 2001). MN assay provide evidence of DNA breakage, spindle, or other parts of the mitotic apparatus dysfunction caused by clastogens and aneuploidogenic poisons (Heddle *et al.*, 1991). The micronucleus induction assay is well-established method that is useful in the evaluation of genotoxic effects of substantial compounds, in fishes (Al-Sabti and Metcalfe, 1995) and other species (Schmid, 1975; Grisolia *et al.*, 2004). MN analysis has been used as an index of cytogenetic damage for many years (Heddle *et al.*, 1991). It is an easy, reliable method, rapidly analysed, inexpensive and an excellent indicator of genotoxicity of chemical contamination in fish employed for laboratory assays (Hose *et al.*, 1987; Marrazzini *et al.*, 1994). MN analysis is employed in both marine and freshwater ecosystems to biomonitor wild areas with different levels of contamination, employing as marker species a variety of organisms, ranging from mussels (Mersch and Beauvais, 1997) to fish (De Flora *et al.*, 1993; Minissi *et al.*, 1996; Hayashi *et al.*, 1998) and amphibian (Fernandez *et al.*, 1993). MN assay has been used in both laboratory and field studies in vertebrates *e.g.*, fishes (*Cyprinus carpio, Gambusia holbrooki, Poecilia latipinna, Salmo trutta,* and *Phoxinus Phoxinus*) (Sanchez-Galan *et al.*, 1999; Ayllon and Garcia-Vazquez,

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2000; Buschini *et al.,* 2004; Russo *et al.,* 2004).

Fish are used for the study of the mutagenic and carcinogenic potential of environmental contaminants present in aquatic samples as they can metabolize, concentrate and store pollutants (Al-Sabti, 1991). Fish and shellfish are susceptible to pesticides pollution (Shoaib *et al*., 2012; Shoaib and Siddiqui, 2015). Fish respond to pollutants similar to higher vertebrates therefore fish (*e.g., Mullus* sp., *Platichthys flesus* L., *Zoarces viviparus, Perca* sp.) are used in monitoring programs as sensitive indicators, so-called sentinel organisms (Krishnakumar *et al.,* 1994). Genotoxic chemicals are liable for DNA damage in marine organisms causing malignancies, reduced survival of embryos, larvae and adults. Genotoxicity reduces the 'fitness' (*i.e.* growth, fertility and fecundity) in fish populations. Besides, causing mortality, these pollutants can cause genotoxicity in aquatic organisms which can lead to development of tumors in fishes (Folmar *et al.,* 1993).

Oreochromis mossambicus is found in tropical and subtropical habitats, live in rivers, lagoons, creeks and streams. *O. mossambicus* is very hardy, euryhaline fish, have a broad salinity and temperature tolerance. The Mozambique tilapia is an invasive species in many parts of the world, having escaped from aquaculture or been deliberately introduced to control mosquitoes (Moyle, 1976). The present study investigates the genotoxic effect of pesticide using MN assay in erythrocytes of fish exposed *in vivo.* The aim of the present study is to assess the MN frequency in the fish *O. mossambicus* peripheral blood erythrocytes after exposure to different concentrations of organophosphate (OP) pesticides (chlorpyrifos, malathion), synthetic pyrethroid (SP) pesticides (cypermethrin, lambda-cyhalothrin) and herbicide (buctril).

MATERIALS AND METHODS

Pesticides were purchased from the market. The OPs (chlorpyrifos 40% EC, malathion 57% EC), SP pesticides (cypermethrin 10% EC, lambda-cyhalothrin 2.5% EC) and herbicide (buctril 60% EC) were used in the fore going study.

Fish

The fishes *O. mossambicus* were collected from Chilya hatchery Thatta. The fish were transported in clear aerated water to the laboratory ensuring minimum stress. Fishes (2.7±1cm) length, (5±1g) weight were used in this experiment. The fishes were acclimatized in the laboratory conditions for 48 h prior to experiments. The fishes were kept in clean aerated seawater in glass aquaria (92 cm length x 39cm width x 47 cm height) at 23±1°C, with photoperiod 16 h of light and 8 h of dark (16 L: 8 D) cycle. Seawater in each aquarium was replenished every day in order to remove faeces and remaining food every day and to maintain the water quality and oxygen saturation level above 60%. Fishes were fed ad libitum and commercial diet two times a day. All the glassware was acid washed prior to the tests (Bellan, 1981) and natural seawater was used throughout the experiments.

Experiments were carried out in glass aquarium (30.5 cm Length x 30.5cm width x 30.5 cm height). All pesticide concentrations were prepared with filtered seawater. Fish were given *ip* injection of five different concentrations of selected pesticides in the trunk muscle. The control group received the same volume of sterilized injection of only seawater. The experiment was performed in triplicate. Three controls were also set up for each experiment. The experiment was performed at 23±1°C, Salinity 30ppt, pH 7.5, photoperiod 16 h light and 8 h dark.

Micronucleus test

The peripheral blood collection was obtained through the gills following dissection. A thin, uniform blood smear was prepared on a clean glass slide. The slides were air-dried for 24h, in a dust-free and moisture-free environment. The slides were fixed in methanol for 10 min, followed by 10% Giemsa (v/v) staining. The test was performed in triplicate (both test and control). From each fish 1000 erythrocytes were examined. To detect micronuclei in erythrocytes, the slides were observed under a light microscope using oil immersion. On each slide, 1000 cells were counted (*i.e.*, 3000 cells per concentration). Only intact cells with distinct nuclear and cellular membranes were scored. Micronuclei (MN) were identified according to the following criteria spheric cytoplasmic inclusions with a sharp contour, diameter smaller than one-third of the nucleus, colour and texture resemble the nucleus, no contact with the nucleus (Tates *et al.,* 1980; Majone *et al.,* 1987; Babich *et al.,* 1990).

The MN frequency was calculated as:

$$\%MN = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

RESULTS

In the present study MN frequencies in the fish peripheral blood erythrocytes after exposure to different concentrations of OP pesticides (chlorpyrifos, malathion), SP pesticides (cypermethrin, lambda-cyhalothrin) and herbicide (buctril) show increase in frequencies as

compared to control group (Figs. 1A-D). The MN frequencies of the OP, SP and herbicide (buctril) treated fish are observed to increase significantly ($p<0.05$) with increase in concentration and time at all exposure periods. The MN frequencies were significantly different from control ($P<0.05$) as compared to pesticides treated groups and the MN frequencies of all the five pesticides treated fish groups continuously increased significantly ($p<0.05$) until the end of the exposure period as compared to control group. The highest MN frequencies were recorded after 48 h cypermethrin exposure (from 0.0001-0.01 ppm, respectively) (Fig. 1C). However, the lowest MN frequencies were recorded after 48 h buctril exposure (from 0.0001-0.01 ppm, respectively) (Fig. 1E). The frequencies of MN formation at 48 h were significantly ($p<0.05$) higher than those at 24 h in all pesticides treated groups. The genotoxicity of pesticides on fish in the present study after 24 h exposure was found to be in the order of cypermethrin, malathion, chlorpyrifos, lambda-cyhalothrin and buctril, in peripheral blood erythrocytes. The genotoxicity of pesticides on fish at 48 h exposure in the present study is found to be in the order of cypermethrin, chlorpyrifos, malathion, lambda-cyhalothrin and buctril, in peripheral blood erythrocytes.

DISCUSSION

The MN assay test in fish erythrocyte is widely used for genotoxicity assessment of marine and fresh water organisms (Hughes and Hebert, 1991; De Flora *et al.*, 1993; Al-Sabti and Metcalfe, 1995; Minissi *et al.*, 1996; Hayashi *et al.*, 1998; Barsiene *et al.*, 2004; Cavas and Ergene-Go, 2005; Barsiene *et al.*, 2006; Napierska *et al.*, 2009). The analysis of MN frequency in the fish erythrocytes has been reported in several studies exposed to pesticides (Campana *et al.*, 1999; Abdul-Farah *et al.*, 2003; Monteiro *et al.*, 2006; Ali *et al.*, 2008; Muranli and Güner, 2011; Kankaya *et al.*, 2012).

The present study reports dose and time dependent increase in MN induction in the peripheral blood erythrocytes of fish (*Oreochromis mossambicus)* which is in line with authors (Hooftman and De Raat, 1982; Bahari *et al.*, 1994; Abdul-Farah *et al.*, 2003; Ali *et al.*, 2008). However, in the present study it was observed that there was a basal level of measurable spontaneous micronuclei formation in *O. mossambicus* which was also observed in most of the fish species (Al-Sabti and Metcalfe, 1995), exposure to clastogens, both in the laboratory and in the field (Bombail *et al.*, 2001; Grisolia and Starling, 2001; Rodriguez-Cea *et al.*, 2003) can elevate the frequency of MN. In the beginning of exposure period the mature (and non dividing)

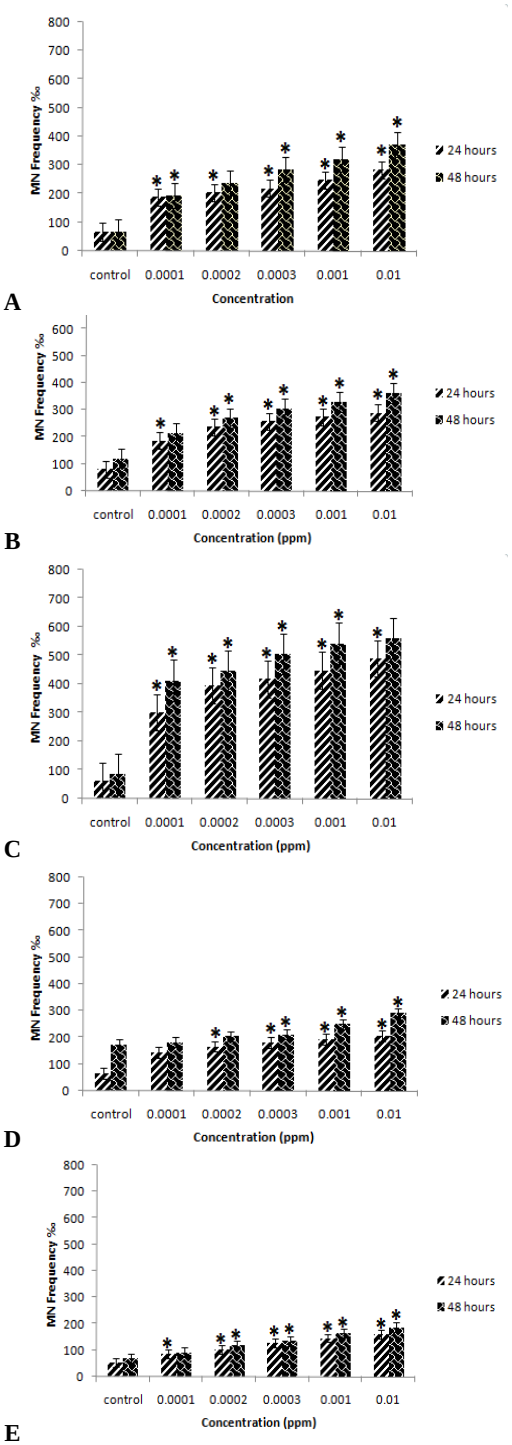

Fig. 1. Micronuclei frequency in peripheral erythrocytes of fish, *Oreochromis mossambicus* after chlorpyrifos (A), malathion (B), cypermethrin (C), lambda-cyhalothrin (D) and Buctril (E) treatments. Bars with asterisk are significantly different from control ($p<0.05$).

erythrocytes predominate in the blood, the detection of induced MN in mature blood cells will be at a low frequency. As with the passage of time, increase in the number of dividing cells (polychromatic erythrocytes) would predominate in the blood (Al-Sabti and Metcalfe, 1995). However, the MN test is a sensitive assay to evaluate genotoxic compounds in fish (Bolognesi *et al.,* 2006), it might suffer variations according to clastogen, test organism, and the life cycle of the cells (Grisolia and Cordeiro, 2000). During MN study intraspecific factors may affect the response in assays include age (Christine and Costa, 1983), sex (Urlando and Heddle, 1990) and diet (Virgano *et al.,* 1993). The MN frequencies may vary with the season, temperature, oxygen, the kind of pollution involved, and the species of fish (Kligerman, 1982; Dixon *et al.,* 2002).

In the present study peripheral blood erythrocytes of fish were used as most of the MN surveys have been carried out in peripheral blood erythrocytes of fish (De Flora *et al.,* 1993; Minissi *et al.,* 1996). Counting of MN is faster and less technically demanding than scoring of chromosomal aberrations, the MN assay has been widely used to screen for chemicals that cause these types of damage. Fish has been considered as an efficient and cost effective for studying the toxic and carcinogenic potential of contaminants (Belpaeme *et al.,* 1996; Spitsbergen and Kent, 2003) due to their ability to metabolize, concentrate, and store water-borne pollutants (Al-Sabti, 1991). MN assay provide information as a simple bioindicator for chromosomal aberrations not available from other methods: (i) the consolidated effect of a variety of environmental stresses on the health of an organism, population, community, and ecosystem (ii) warning of harmful effects to human health based on the responses of wildlife to pollution, and (iii) the effectiveness of remediation efforts in decontaminating waterways (Villela *et al.,* 2006).

The present results demonstrate that MN test in fish can be used for the genotoxicity assessment in a marine environment. Fish are considered as sentinel organisms in a health assessment of an aquatic environment (Dixon *et al.,* 2002; Van der Oost *et al.,* 2003). Mersch *et al.,* (1996) demonstrates that MN frequencies are widely affected by experimental factors, such as the histological method used, the staining method selected, the criteria for the scoring of MN, the test chemicals and concentrations used and the exposure period. The toxic pesticides used, concentrations and the exposure period may be the reason for relatively high MN frequencies recorded in our pesticide treated fish.

In the foregoing investigation on the genotoxicity of the OP pesticides (chlorpyrifos, malathion), SP pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril) suggested a serious apprehension about its potential danger to *O. mossambicus*, and subsequently to human beings by food chain. However, *O. mossambicus* is hardy fish and any impact of pesticides would indicate much more impact on other susceptible species. There is a need for further studies to explore the consequences of DNA damage in marine organisms after pesticides exposure and to formulate the future strategies for safeguarding marine environment. The results of the present study show that the assay can be employed for the evaluation and the assessment of water pollution and aquatic mutagens.

Statement of conflict of interest

Authors have declared no conflict of interest.

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