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Genotoxic Effect of Pesticides on *Perna viridis*

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ABSTRACT

Genotoxicity is considered as one of the endpoints in assessing toxic effect of pollution. In the present study genotoxic effects of pesticides in the *Perna viridis* have been evaluated by the induction of micronuclei (MN). The aim of the present study was to assess the MN frequency in the haemolymph of green mussel (*Perna viridis*) after exposure to different concentrations of organophosphate pesticides (chlorpyrifos, malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril). Haemocytes of bivalve play an important role in immune defense and detoxification of contaminants. The bivalves were exposed to different concentrations of test pesticides in a static system. The MN frequencies of all the pesticide treated mussel groups increased significantly ($p< 0.05$) until the end of the exposure period as compare to control. The highest MN frequencies were recorded after cypermethrin exposure on day 12 (7, 8.5 and 11‰ for 0.5 ppm, 1 ppm and 1.5 ppm concentrations respectively) in haemolymph. However the lowest MN frequencies were recorded after buctril exposure (3.5, 3.5 and 5‰ for 0.5 ppm, 1 ppm and 1.5 ppm concentrations, respectively) in haemolymph. The use of haemocytes of green mussel (*Perna viridis*) in MN assay proved to be a sensitive tool for the assessment of genetic damage.

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Key words
Genotoxic, Organophosphate, Pesticides, Bivalve, Haemolymph.

INTRODUCTION

Wide varieties of pesticides are used for crop protection against pest in Pakistan at the same time they have adverse effects on environment. Pollution is a major threat to marine organisms, especially bivalves living in coastal environments. Mussels have great commercial and nutritional value. Mussels have been considered as bioindicator organisms because of their wide geographical distribution, and sensitivity to environmental pollutants. They are sessile, filter feeders as they accumulate and concentrate pollutants within their tissues (Bernal-Hernandez *et al*., 2010). The mussel has been used as a sentinel species in many biomonitoring research programmes (Widdows *et al*., 1995; O'Connor, 1996; Dixon *et al*., 2002). Pollutants in marine environment can adversely affect DNA of filter-feeding mollusks (Hamoutene *et al*., 2002). Micronucleus (MN) is considered as the marker of cytogenetic damage, appearing after the impact of genotoxic compound. Micronuclei are made from piece or whole chromosomes that lag in cell division due to defect in cytokinesis. These small secondary structures of chromatin, present in the cytoplasm form a small nucleus (Seelbach *et al*., 1993; Zoll-Moreux and Ferrier, 1999). In marine organisms due to genotoxicity, stunted growth, infertility and sterility have been reported at the same time have harmful effects on human health by food chain. In marine organisms genotoxic effect cause mortality and sometimes development of tumors takes place (Folmar, 1993).

Micronucleus test is one of the most popular and promising tests (Fenech *et al*., 2003). It is considered as a sensitive, fast and extensively used method in the detection of genotoxicity of chemical substances in the environment (Mersch and Beauvais, 1997; Kassie *et al*., 2000). Moreover, the endpoint is easily recognizable, and scoring of MN can be done considerably faster (Siu *et al*., 2004). The MN test is one of the best biological marker that relates with pollution load, and has been shown by many studies (Bolognesi *et al*., 2006a; Baršienė *et al*., 2004, 2006).

Micronuclei analysis is used to assess in both marine and freshwater organisms, fewer studies on marine invertebrate organisms have been carried out (Dixon *et al*., 2002). MN assay has been used in both laboratory and field studies in vertebrates *e.g.* fishes (*Cyprinus carpio, Gambusia holbrooki, Poecilia latipinna, Salmo trutta, Phoxinus Phoxinus*, *Ictalurus nebulosus* and *Oreochromis mossambicus*) (Hai *et al*., 1997; Sanchez-Galan *et al*., 1999; Ayllon and Garcia-Vazquez, 2000; Buschini *et al*., 2004; Russo *et al*., 2004; Naqvi *et al*., 2016), and different species of invertebrates *e.g.* bivalves (*Anodonta cygnea*, *Crassostrea corteziensis, Crassostrea gigas*, *Mytilus galloprovincialis*, *Mytilus edulis*, *Mya arenaria, D. polymorpha* and *Perna viridis*) (Burgeot *et al*., 1995, 1996; Dopp *et al*.,1996; Mersch and Beauvais, 1997; Jha *et al*., 2006; Eskandari *et al*., 2012; Benitez-Trinidad *et al*., 2014; Ali *et al*., 2018).

Haemocytes have been extensively used for MN assay (Kalpaxis *et al*., 2004; Baršiene *et al*., 2006). For MN assay, haemocytes are reliable, sensitive, and reproducible

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(Pavlica *et al*., 2000; Klobučar *et al*., 2003; Jha *et al*., 2005). Haemocytes has an important role in uptake of contaminants (Galtsoff, 1964), transportation to various tissues (Cunningham, 1979), and its final elimination. Haemocytes are responsible for the transportation of pollutants from the organ of entry *i.e.,* gill and mantle to tissues where detoxification or accumulation takes place (Ruddel and Rains, 1975; George *et al*., 1978).

The aim of the present study is to assess the MN frequency in the haemolymph of green mussel (*Perna viridis*) after exposure to different concentrations of organophosphate pesticides (chlorpyrifos, malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril).

MATERIALS AND METHODS

Insecticides used

Pesticides were procured from the market organophosphate (chlorpyrifos 40% EC, malathion 57% EC) and synthetic pyrethroid pesticide (cypermethrin 10% EC, lambda-cyhalothrin 2.5% EC) and herbicide (buctril 60% EC) were used.

Experimental design

The bivalves *Perna viridis* (shell length 5-6 cm) were collected from rocky shore of Manora by handpick method. The bivalves were transported in coolbox safely to the laboratory. The bivalves *Perna viridis* were cleaned to remove biofouling organisms and acclimatized in the laboratory condition at room temperature for three days. Temperature 26±2°C, salinity 35±2 ppt and pH 7.4 of seawater were maintained in aquarium (92 cm Length x 39 cm width x 47 cm height). Seawater in aquarium was replenished every day in order to remove faeces and to maintain the water quality. The acclimatized bivalves of approximately same size were selected for experiment. The bivalves were exposed to different concentrations (0.5ppm, 1ppm and 1.5 ppm) of test pesticides in a static system. The test water was replenished every day during the experimentation and proper oxygenation in the test solution was ensured. The tests and controls for each experiment were in triplicate and the controls had only seawater. The other experimental conditions, such as, temperature 26±2°C, salinity 35±2 ppt and pH 7.4 were maintained throughout the experiment. The physico-chemical parameters of the test water were analyzed using standard methods (APHA, AWWA, WPCF, 2005). From each group, the tissue samplings were done on days 1, 4, 8 and 12 after pesticide exposure for performing the MN test. Haemolymph were used for MN analysis.

Micronucleus test

The micronucleus analysis in haemolymph of bivalves method of Siu *et al*. (2004) was followed. Haemolymph from individual mussels was collected from the posterior adductor muscle using a 1ml hypodermic syringe. The haemolymph was dropped on to a clean microscopic slide and air-dried completely at room temperature (26±2°C). The slides were fixed in methanol for 1 min at room temperature and stained with 10% Giemsa in phosphate buffered saline, pH 6.6 for 10 min. The slide was then dried. Four replicate slides per specimen were prepared for every sampling time. The slides were observed under a light microscope using oil immersion (2000 cells from each specimen were examined for the presence of MN). The criteria used for the identification of MN were as described by Tates *et al*. (1980) (their size lesser than one-third of the main nucleus, no attachment with the main nucleus, same color and intensity as the main nucleus). The result was calculated as MN frequency using the following formula: % of X = (number of X/2,000) x 1,000, where X is MN (Montero *et al*., 2006). The results obtained for each experiment, for control and for test group were compared with each other using two-tailed Student t-test. Differences between means were considered significant when $P < 0.05$.

RESULTS

The MN frequencies of haemocytes of mussels of the control group is observed to be low (ranging from 0.0 to 1.5‰) as compared to the pesticides exposed groups in all the five pesticides treated mussels (Fig. 1). The MN frequencies of the pesticides treated mussels are observed to increase significantly ($p<0.05$) in a dose-dependent relationship at all exposure periods. The MN frequencies of all the pesticide treated mussel groups continuously increased significantly ($p<0.05$) until the end of the exposure period as compare to control. The highest MN frequencies were recorded after cypermethrin exposure on day 12 (7, 8.5 and 11‰ for 0.5 ppm, 1 ppm and 1.5 ppm concentrations, respectively) in haemolymph. However the lowest MN frequencies were recorded after buctril exposure (3.5, 3.5 and 5‰ for 0.5 ppm, 1 ppm and 1.5 ppm concentrations, respectively) in haemolymph. The frequencies of MN formation at day 12 were significantly ($p< 0.05$) higher as compare to, day 1, 4 and 8 in all pesticides treated mussels. The genotoxicity of pesticides on *Perna viridis* in this study is found to be in the order of cypermethrin, chlorpyrifos, malathion, lambda-cyhalothrin and buctril, in haemolymph.

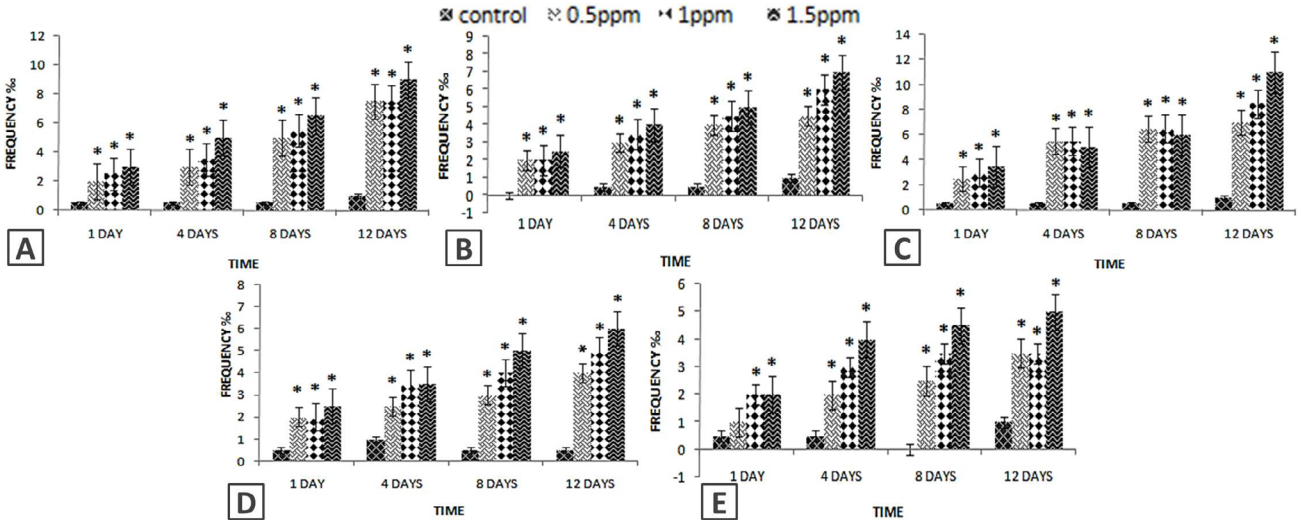

Fig. 1. Effect of chlorpyrifos (A), malathion (B), cypermethrin (C), cyhalothrin (D) and buctril (E) on micro nuclei of haemolymph of *Perna viridis.*

DISCUSSION

The analysis of MN in haemocytes of mollusk (*Anodonta cignea*, *Crassostrea gigas*, *Mytilus galloprovincialis*, *Mytilus edulis* and *Mya arenaria*) has been reported in several studies (Scarpato *et al*., 1990; Bolognesi *et al*., 1992, 1996; Wrisberg *et al*., 1992; Burgeot *et al*., 1995, 1996; Dopp *et al*., 1996). In the present study the MN frequencies of heamocytes of mussels after exposure to organophosphate pesticides (chlorpyrifos, malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril) are studied. The MN frequency of the pesticides treated mussels are observed to increase in a dose-dependent relationship at all exposure periods. Similar results were observed by Bolognesi *et al*. (1996), Venier *et al*. (1997), Jha *et al*. (2005), Koukouzika and Dimitriadis (2008) and Ali *et al*. (2018).

The open vascular system of the mussels is composed of haemocytes (Mersch *et al*., 1996) and can be used for observing cytogenetic damage (Pavlica *et al*., 2001). Different fish and shellfish cell types were used for the MN analysis such as gill, fin, kidney, hepatic cells and peripheral erythrocytes (Al-Sabti and Metcalfe, 1995). However in the foregoing study we use haemocytes for MN assay in *Perna viridis.* Haemocytes are most frequently used for MN assay in bivalve (Kalpaxis *et al*., 2004; Baršiene *et al*., 2006). Haemocytes are the target tissues considered for MN determination in bivalves (*Perna viridis*) as they constitutes a massive part of a mussel's soft tissue, as circulating cells of an open vascular system, and are continuously exposed to contaminants. Haemocytes perform several functions for example elimination of noxious substances and small particles, transport and digestion of nutrients and restoration of tissue lesions (Soares-da-Silva et. al., 2002), they represent cells of significant importance in the mussel's response to pollutants.

In the present study the MN frequencies of haemocytes of mussels after exposure to organophosphate pesticides, synthetic pyrethroid pesticide and herbicide shows significant genotoxic effect and increases with the passage of time. Bolognesi *et al*. (2006b) concluded that magnitude of the genotoxic response is correlated with the duration of exposure and age is considerably related with the increase in occurrence of MN. In addition, haemolymph readily provides a single-cell suspension, it is easily collectable and its usage permits repeatable tissue sampling of the same individuals for the MN assay. Therefore haemocytes are used in bio-monitoring as they provide a relatively non-invasive source of material (Fossi *et al*., 1994; Mitchelmore and Chipman, 1998; Taddei *et al*., 2001) and they can be quickly and easily sampled and cell dissociation is not required so they are appropriate for MN assay (Belpaeme *et al*., 1998). The extent of proteolytic cell detachment and artificial cellular damage from mechanical source is minimum. These properties short the time for slide preparation and facilitate sample processing. Moreover, haemocytes play an important part in immune defense, phagocytosis, and detoxification of xenobiotics (Cheng, 1975); their multifunctional work has been suggested to make them more vulnerable than other cells towards contaminants such as the genotoxic xenobiotics (Venier *et al*., 1997).

The results of our study show that MN test in mussels can be used for the genotoxicity assessment in a marine environment. This is in agreement with earlier and recent studies by other researchers (Hose *et al*., 1987; Al-Sabti and Hardig, 1990; Al-Sabti and Metcalfe, 1995; Ayllon and Garcia-Vazquez, 2000; Bombail *et al*., 2001; Dailianis *et al*., 2003; Naqvi *et al*., 2016; Ali *et al*., 2018). According to Mersch *et al*. (1996) MN frequencies are strongly affected by experimental factors, such as the histological method selected, the staining method used, the criteria for the scoring of MN, the test chemicals and concentrations used and the period of exposure. The last two factors may be the reason for relatively high MN frequencies recorded in our pesticide treated mussels.

The number of MN formation in different organisms may be different as organisms respond to stress differently due to transcription of specific genes, or different levels of absorption and metabolisms of genotoxic agents, DNA repair, cell death and defense mechanisms (Pavlica *et al*., 2000; Rodriguez *et al*., 2003). These differences are connected to cell removal kinetics or to the development of adaptive mechanisms of tolerance to chemical stress that help an increase in the replacement rate of dead cells to maintain normal physiological conditions or inhibition of nuclear division which is required for MN expression (Cavas and Ergene-Gozukara, 2005). During the current research it was observed that green mussel *Perna viridis* represent variation in sensitivity towards the organophosphate pesticides (chlorpyrifos, malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril). Although organophosphate, pyrethroid pesticides and herbicide are not persistent in the environment but continuous use of these pesticides may have hazardous effect on marine environment, on marine organisms and subsequently to human health, due to bioaccumulation.

Marine pollution possesses a threat to the ecological balance of the marine environment and biodiversity (Vosyliene and Jankaite, 2006). Present study clearly reveal the genotoxic potential of pesticides and suggest a serious concern towards the potential danger of pesticide to aquatic organisms and its use in agriculture practices.

CONCLUSION

Pesticides are widely used for controlling pest for increasing production of agricultural products, vegetables, food grain and to control insects' vectors of diseases. The development of MN in haemocytes of mollusk in the present study after exposure to pesticides clearly indicates genotoxic nature of pesticides. Micronucleus studies have received considerable attention in recent years, from a growing interest in the evaluation of genotoxicity of environmental toxicants and carcinogens. Current study clearly demonstrates Micronucleus assay test can be used for the genotoxic effects as the main biomarkers in assessment of pollution related toxicity.

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Statement of conflict of interest

The authors have declared no conflict of interests.

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