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Genotoxic effect of pesticides on gill tissues of green-lipped mussel *Perna viridis* (L.)

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The marine ecosystem is constantly threatened by a wide variety of anthropogenic hazardous chemicals, such as, heavy metals, pesticides, oil, petroleum hydrocarbons, etc., from industries, agricultural sources and sewage disposal. Pakistan, being a country with agriculture prominence, uses pesticides widely for crop protection, and thereby suffers from pollution. In the present study, we assessed a few biomarkers as indicators of the genotoxic chemicals, pesticides and herbicides. We inducted micronucleus (MN) in the gill tissues of green mussel *Perna viridis* (L.) exposed to different concentrations of organo-phosphate pesticides (chlorpyrifos, malathion) and synthetic pyrethroid pesticides (cypermethrin, lambda-cyhalothrin) and a herbicide (buctril). The MN frequencies of the pesticides treated mussels were observed to increase significantly (P <0.05) in a dose-dependent manner at all exposure periods. The highest MN frequencies were recorded in gill tissues of cypermethrin treated mussels on the 12th day (10, 11.5 and 13.5‰ at 0.5, 1 and 1.5 ppm, respectively). The genotoxic effect of pesticides on *Perna viridis* (gill tissue) was in the following order cypermethrin > chlorpyrifos > malathion > lambda-cyhalothrin > buctril.

Keywords: Buctril, Chlorpyrifos, Cyhalothrin, Cypermethrin, Herbicides, Malathion, Micronucleus, Mussels, Pollution

Pesticide pollution is a potential threat to aquatic organisms, especially sedentary organisms, for example, bivalves living in coastal and riverine environments. Mussels (bivalvia) are sedentary filter feeders and bioaccumulate pollutants within their tissues[1-3]. The mussel has been used as a sentinel species in many biomonitoring research programmes[2-4]. Bivalve mollusks have great commercial importance and consumed worldwide as food which makes it more important to monitor safety of these fishery products for human consumption. Several chemical compounds can adversely affect DNA of filter-feeding mollusks[5]. For example, induction of micronucleus (MN) as a result of exposure to genotoxic chemicals is being used as marker of cytogenetic damage. Micronuclei are produced from fragments or entire chromosomes that lag in cell division because of a lacking or damaged centromere or a defect in cytokinesis. These small secondary structures of chromatin, present in the cytoplasm and surrounded by membranes, have no attachment to the cell nucleus[6]. Genotoxicity affects growth, fertility and fecundity, develop tumors and causes mortality in marine organisms[7] and at the same time poses risk for human health. Assessment of micronucleus (MN) formation is an extensively used, sensitive and efficient method employed for the detection of genotoxicity of chemical substances present in the environment[8-10]. Moreover, the endpoint is easily recognizable, and scoring of MN can be done considerably faster[11].

Micronucleus assay has been used in the detection of genotoxicity of various pollutants (polycyclic aromatic hydrocarbons, organophosphate pesticides, synthetic pyrethroid, chlorinated pesticides, herbicides, heavy metal, tritium, benzo[a]pyrene, mitomycin C and colchicines), for example, in fishes (*Channa punctatus* (Bloch). *Oreochromis mossambicus, Cirrhinus mrigala*)[10,12,13] and bivalves (Clams, *Lamellidens marginalis*, *Mytilus* spp., *Perna viridis* and *Septifer virgatus*)[3,11,14-17]. The bivalve mollusks are exposed to pollutants mainly through gill, mantle and during filter feeding activity[17], and therefore, gill cells have been extensively used for MN assay[4].

In Pakistan, a wide variety of chemicals, such as, pesticides (organophosphates: chlorpyrifos, malathion and synthetic pyrethroids: cypermethrin, lambda-cyhalothrin) and herbicide (buctril)) are used extensively for crop protection and their passage to the natural waters poses threats to the living organisms. Biomarkers for easy and quick detection of the impact of genotoxic chemicals on environmental health are required. Therefore, in the present study, we examined induction of micronuclei (MN) in green mussel *Perna viridis* (L.) exposed to different concentrations (0.5-1.5 ppm) of pesticides (organophosphates: chlorpyrifos, malathion and synthetic pyrethroids: cypermethrin, lambda-cyhalothrin) and a herbicide (buctril).

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Materials and Methods

The bivalves *Perna viridis* (shell length 5-6 cm) were handpicked from rocky shore at Manora Island, Karachi, Pakistan. Live samples were brought to the laboratory, cleaned to remove biofouling organisms and acclimatized in aerated seawater contained in glass aquarium (L×W×H: 92 × 39 × 47 cm) for three days. The water parameters, such as, temperature (26±2°C), salinity (35+2 psu) and pH (7.4) were maintained. Seawater was replenished every day in order to remove feces and to maintain the water quality. The active and healthy animals of approximately same size were selected for the experiment. The bivalves were exposed to different concentrations (0.5, 1 and 1.5 ppm) of test pesticides in a static system where aeration was ensured and water was replenished every day during the experimentation. Experiments were set in small aquariums in triplicate for each pesticide and controls (seawater only) having ten animals each. The physicochemical parameters of water were analyzed regularly using standard methods[18] and maintained; temperature (26±2°C), Salinity (35+2 psu) and pH (7.4). One animal was picked from each aquarium set in triplicate for each pesticide/control and gill tissue samples were obtained on 1st, 4th, 8th and 12th day after pesticide exposure for the analysis of MN induction.

Micronucleus (MN) assay

For the analysis of MN induction in gill tissues of bivalves, modified method[11,21] was followed. Gill tissues were washed in phosphate buffered saline (PBS; pH 6.6) and placed on a clean slide in a drop of PBS (pH 6.6), and gently nipped with tweezers for 2-3 min (until cells spread within a drop). Tissue debris was removed and the cell suspension was softly smeared on the surface of a slide. The slides were air-dried completely, fixed in methanol for 1 min at room temperature (27±2°C), stained for 10 min with 10% Giemsa in PBS and air dried. Four replicate slides per specimen were prepared for each tissue sample. The slides were observed under a light microscope using oil immersion (2000 cells for each sample were examined for the presence of MN).

Micronuclei (MN) were identified using criteria described earlier by Tates *et al*.[20], for example, (size smaller than one-third of the main nucleus, no attachment with the main nucleus, same colour and intensity as the main nucleus, etc.). The result was calculated as MN frequency using the following formula: ‰ of MN = (number of MN/2000) × 100[21]. The results obtained for controls and for test group, for each experimental period, were compared with each other using two-tailed Student t-test. Differences between means were considered significant when $P < 0.05$.

Results and Discussion

The results of the present study are summarized in Fig. 1 A-E. The results depict that the green mussel (*Perna viridis*) represent variation in sensitivity towards organophosphate pesticides (chlorpyrifos, malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril). The

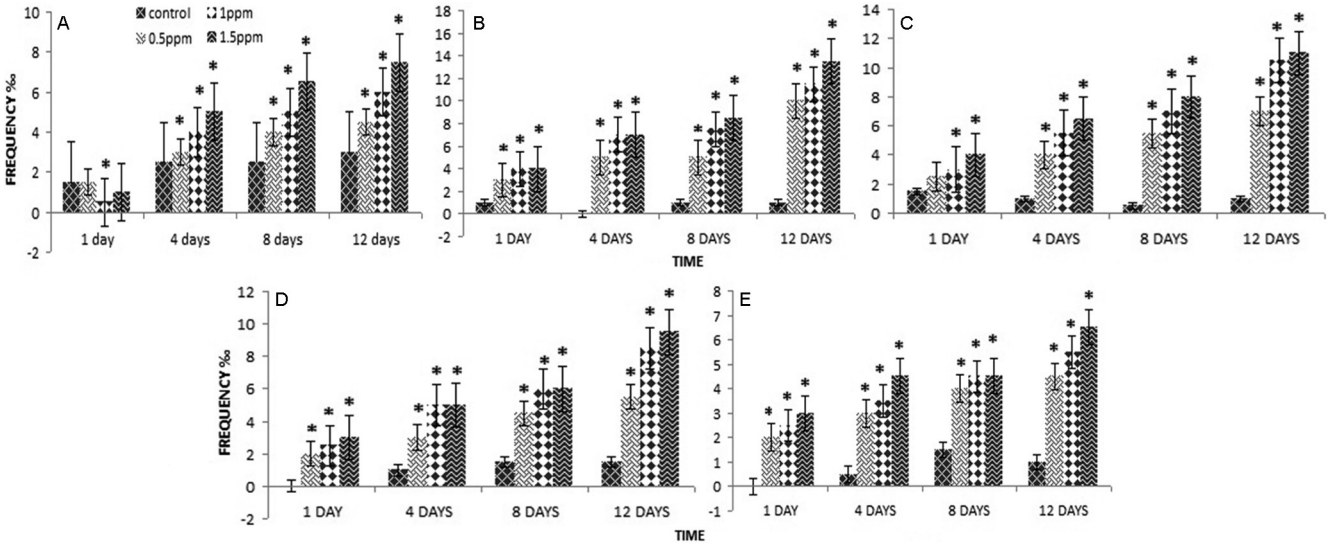

Fig. 1 — Effect of (A) Lambda-cyhalothrin; (B) Cypermethrin; (C) Chlorpyrifos; (D) Malathion; and (E) Buctril on MN of Gills of *Perna viridis* L. [Bars with asterisk are significantly different from control ($P < 0.05$)]

sensitivity of *Perna viridis* towards five pesticides was obvious by the formation of MN representing genetic damage (clastogenic effect). The micronucleus (MN) spontaneous frequency in the control group was low (0.0-1.5‰) as compared to the treated groups (Fig. 1 A-E). The MN frequencies in pesticides treated mussels were increased significantly (P <0.05) in a dose dependent manner at all exposure periods. The highest MN frequencies were recorded in mussels exposed to cypermethrin on day 12 (10, 11.5 and 13.5‰ at 0.5, 1 and 1.5 ppm, respectively) (Fig. 1B). The second highest MN frequencies were recorded in chlorpyrifos exposed mussels on day 12 (7, 10.5 and 11‰ for 0.5, 1 and 1.5 ppm concentrations, respectively) (Fig. 1C), followed by malathion (5.5, 8.5 and 9.5‰ at 0.5, 1 and 1.5 ppm, respectively) (Fig. 1D), lambda-cyhalothrin (4.5, 6 and 7.5‰ for 0.5, 1 and 1.5 ppm, respectively) (Fig. 1A) and buctril (4.5, 5.5 and 6.5‰ for 0.5, 1 and 1.5 ppm, respectively) in gill tissue (Fig. 1E). The MN frequencies recorded at day 12 were significantly (P <0.05) higher than those observed at day 1, 4 and 8 in all pesticides treated mussels.

The analysis of MN in different species of mollusk (*Anodonta cignea*, *Crassostrea corteziensis*, *Dreissena polymorpha*, *Lamellidens marginalis* and *Unio pictorum*) has been reported[3,14,22-25]. In the present study, an increase in MN frequencies in the gills of mussels (*Perna viridis*) after exposure to clastogens, organophosphate pesticides (chlorpyrifos, malathion), synthetic pyrethroid pesticide (cypermethrin, lambda-cyhalothrin) and herbicide (buctril) are reported. Similar results were observed by Guidi *et al.*[25] (*Unio pictorum* exposed to polluted sediments); Jha *et al.*[26] (*Mytilus edulis* exposed to tritium); Koukouzika & Dimitriadis[27] (mussels exposed to phenanthrene, Cu, Cd, and Hg); and Siu *et al.*[11] (*Perna viridis* exposed to mixtures of polycyclic aromatic hydrocarbons and chlorinated pesticides). The significant (P <0.05) increases in MN frequency with increasing pesticide concentration and exposure period in all treated mussels observed in the present study, is in agreement with the earlier reports[11,26-28].

It is evident from our results and previous studies that MN frequency varies from organism to organism and with type of pesticides tested. The MN frequency (6.5‰ (buctril)-13.5‰ (cypermethrin); 1.5 ppm; 12 d) observed in *P viridis* gill tissues exposed to five different clastogens was within the range that has been observed in gill tissues of freshwater zebra mussels (2 and 12‰) exposed to four different clastogens for 12 days[28]. However, fish appear to have low response to pesticide exposure as reported in many studies. In *Chalcalburnus tarichi*, MN frequency ranged from 6.35-17.23‰ exposed at 1.47-6.11 ppm of methyl parathion[29]. In *Channa punctatus* exposed to pentachlorophenol (PCP), Farah *et al.*[30] observed MN frequency between 2.89-8.08‰ at 0.6 ppm. They also reported MN frequency 1.96‰ after 48 h in *Channa punctatus* exposed to 2,4-D (2,4-dichlorophenoxyacetic acid) to 4.62‰ at 96 h.

Most bivalve species have been reported to possess relatively higher spontaneous MN frequencies (>1‰)[28]. For example, the zebra mussel (*Dreissena polymorpha*), a freshwater mussel (*Anodanta cygnea*), had mean spontaneous MN frequencies of 2.7 to 3.4‰ [23,28] which is much higher than that of *Perna viridis* (0-1.5‰) observed in the present study and reported by Siu *et al.*[11] and in other bivalves[15]. The lower spontaneous frequency of MN formation in the present study may be attributed to inherently lower spontaneous rates of formation in *P. viridis* or due to the improved experimental procedure.

We used gill cells for MN assay as gills are among the main target organs for bioaccumulation and show the highest pollutant levels in mussels[3,11], play an important role in the feeding, transportation and capture of particles[16], and act as main barrier against environmental pollutant injury and pathological agents[31]. Gill cells, being in direct contact with external environment, are considered as an ideal tissue type for MN assay[15,23,28]. Gill tissues are preferred as they are composed of homogenous cell type and can be easily prepared into a single cell suspension without the requirement for chemical dissociation in Comit assay[32]. According to Hayashi *et al.*[33] the study of micronuclei in gill cells is a more sensitive technique than the analysis of micronuclei in peripheral blood.

In agreement with the previous studies[25,34,35], the present data reveals that MN method in mussels can be used as tool in the assessment of genotoxicity in marine environment. According to Mersch *et al.*[28], MN induction is 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 *P. viridis* in the present study.

The degree of sensitivity of *P. viridis* against different pesticides and herbicide tested was variable

and the genotoxicity of cypermethrin was highest in the gill muscles followed in descending order by, chlorpyrifos, malathion, lambda-cyhalothrin and buctril. Although these pesticides and herbicide are not persistent in the environment, their continuous use may have hazardous effect on marine environment and marine organisms. Bioaccumulation of such clastogens in fish and mussels[36-38], will subsequently reach to other organisms at higher trophic levels and also pose risk to the human health.

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