

***Mugil cephalus* Genome: A Sensitive Monitor for Genotoxicity and Cytotoxicity in Aquatic Environment**

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Abstract: The precise detection of genotoxic effects induced by environmental contaminants is an essential part of any genetic toxicology screening programme. Fish, however, provides a sensitive monitor for environmental genotoxicity in aquatic environment. *Mugil cephalus* genome has now been employed in this study for detection of clastogenic and cellular lesions induced by aquatic contaminants in Abu-Qir Bay. Assessment of biological marker at chromosome level in fish, micronucleus test, binucleate as well as the study of abnormal shape of nucleus is a suitable measure, in which the presence or absence of genotoxins can be detected in water. In this study, chromosomal aberrations (CAs), micronuclei (MN), nuclear buds (NB), binucleate cells (BN), and fragmented-apoptotic cells (FA) were analyzed in blood, kidney and gill cells of flathead mullet (*Mugil cephalus*) which were collected from four different locations along Abu-Qir Bay (hot spot). The data obtained revealed that the tested locations display differential environmental stresses. The location no. 4, El-Tabia pumping station, was proven to be highly polluted, since all tested types of alterations were observed. The genotoxic bioassays employed in this work showed that *Mugil Cephalus* genome is a sensitive biomonitor for genotoxic as well as cytotoxic damage induced by aquatic contaminants. However, differential environmental stresses detected by other researchers in Abu-Qir Bay area are confirmed by the results obtained from this work.

Key words: Chromosome aberrations, genotoxicity, micronuclei, mullet, nuclear abnormalities.

INTRODUCTION

The demand for a clean and safe supply of water for drinking, agriculture, and recreation has rapidly increased over the last few decades'. With pollution from land-based discharges, coastal industries, municipal outflows, agricultural activities, oil and gas industry (drilling and transportation) and transport activities, thousands of chemicals are entering the marine environment. A large proportion of these compounds are potentially toxic, genotoxic and carcinogenic to aquatic organisms (Jha, 2004& 2008). The interaction of genotoxic contaminants with DNA causes various genetic disturbances, which often are irreversible and can be transmitted to the next generations (Depledge and Fossi, 1994; Dixon *et al.*, 1999; Jha, 2004). The analysis of environmental genotoxicity provides early warning signs of adverse long-term effects of contamination.

Fishes are the most useful bioindicators and erythrocytes of fishes are potential biomarker for in situ monitoring of water quality of an aquatic ecosystem because of their high nutritive value. Genotoxic effect in fish is a matter of great concern because of their potential risk on human health after consumption. Among all other tissues, blood is a suitable biomarker where damages can be detected at gene level due to direct contact with toxicants (De Flora *et al.*, 1993).

A number of biomarkers have been applied for assessment of environmental genotoxicity and the micronucleus (MN) test, as one of the most popular approaches, has served as an index of cytogenetic damage for more than 30 years (Schmid, 1975; Heddle *et al.*, 1991). The assay is well elaborated and allows to evaluate the genotoxicity of compounds at low concentrations and to assess dose-response relationships of both DNA reactive and non-DNA reactive genotoxins (e.g. aneugens) (Al-Sabti *et al.*, 1994; Pacheco and Santos, 2002; Gravato and Santos, 2003; Teles *et al.*, 2003). Micronuclei arise from chromosome fragments or whole chromosomes that lag at cell division due to lack of centromere, damage in centromere region, or defect in cytokinesis (Fenech, 2000). MN is small, secondary structures of chromatin, are surrounded by membranes,

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located in the cytoplasm and have no detectable link to the cell nucleus (Heddle *et al.*, 1991). The MN test has successfully been applied in fish for the assessment of marine genotoxicity in situ (Hayashi *et al.*, 1998; Cavas and Ergene-Gozukara, 2003, 2005a; Baršienė *et al.*, 2004, 2005, 2006a, b; Bolognesi *et al.*, 2006a; Kohler and Ellesat, 2008).

In recent years, growing attention has been paid to nuclear abnormalities other than MN and it was demonstrated that these nuclear abnormalities can serve as an index of genotoxic and cytotoxic damage (Ateeq *et al.*, 2002; Dolcetti and Venier, 2002; Gravato and Santos, 2002, 2003; Pacheco and Santos, 2002; Baršienė *et al.*, 2006a; Bolognesi *et al.*, 2006b; Baršienė and Andreike-naite, 2007). Nuclear buds are morphologically similar to micronuclei with the exception that they are joined to the nucleus by a thin nucleoplasmic connection. It is suggested that nuclear buds arise from the elimination of amplified DNA (Miele *et al.*, 1989; Shimizu *et al.*, 2000) and possibly of DNA-repair complexes (Haaf *et al.*, 1999). Studies performed by Lindberg *et al.* (2007) revealed that in normal and folate-deprived human lymphocytes, the majority of nuclear buds originate from interstitial acentric chromosomal fragments; some of them are formed from terminal chromosome fragments or whole chromosomes, possibly representing nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division or excess DNA. Though, most of the nuclear buds have different origin than micronuclei, studies in human tumor cells have indicated, that in the S-phase of the cell cycle amplified chromosomal or double minute DNA may form buds, later disintegrate from the nucleus and become a micronucleus (Shimizu *et al.*, 2000; Yankiwski *et al.*, 2000).

Fragmentation of cells and apoptosis can be provoked by a variety of factors, including those with genotoxic potential (Steinert, 1996; Baršienė *et al.*, 2006a), or directly associated to cytotoxic events (Bolognesi *et al.*, 2006b). Data on other nuclear abnormalities present a wider view on cellular processes following the exposure and permits the measurement of important complementary genotoxicity and cytotoxicity events.

The assessment of cytogenetic damage has been presented as a very important assay in identification of pollution hazards in marine environment (Dixon *et al.*, 2002). The aim of the current study was to evaluate genotoxic and cytotoxic effects of environmental pollution in selected polluted area of the hot spot, Abu-Qir Bay, (UNEP/WHO 1999). The assessment of micronuclei, binucleate cells, nuclear buds and chromosomal aberration (genotoxicity endpoints) and fragmented-apoptotic cells (cytotoxicity endpoint) was performed in mature peripheral blood erythrocytes, gills and kidneys of the flathead mullet (*Mugil cephalus*).

MATERIALS AND METHODS

Study area:

The hot spot (Abu-Qir Bay):

Abu-Qir Bay was considered one of the profitable grounds for fishes before it was polluted by disposal of sewage and industrial effluents. It is a semi-circular basin which lies at about 35 km northeast of Alexandria city, between longitude 30° 4' and 30° 20' East and latitude 31° 16' and 31° 28' North. Its total area is about 500 km². The bay has a shoreline length of about 50 km. It is relatively shallow with a depth ranging from less than one meter along the coast, increasing gradually away from the shore to reach a maximum depth of about 15m. The slope of the beach is gentle backed by a belt of sand dunes. The bay is bordered from the west by Abu-Qir Peninsula and from the East by Rosetta Peninsula where the Rosetta branch of the River Nile flows into the sea (Moussa, 1973). Abu-Qir Bay is considered as one of the major hot spots along the Mediterranean coast (UNEP/WHO, 1999). It receives annually about 370 × 10⁶ m³ of untreated sewage and industrial waste discharged to the bay through El-Tabia pumping station. The industrial wastes include fertilizers, food processing, textile, paper, dyes and weaving industries (Fig. 1), the annual flux of bulk metals was estimated to be 444.8 tons to the bay and it was observed that the major discharged metals into the bay were copper, zinc and iron (Moussa, 1973).

Sample Collection:

The grey mullet (*Mugil cephalus*) is a local species and an important commercial fish on which the majority of the Egyptian population feed. It is an endemic species (not migratory) which is perfect to be an indicator of its environment. It is a fatty fish, which has the ability to accumulate heavy metals and other pollutants on a greater way than the non-fatty ones (Ferreia, *et al.*, 2004). Four different polluted locations were chosen and *Mugil cephalus* fish (grey mullet) were caught from these locations and used for the cytogenetical analysis. These locations are shown in fig. (1).



Fig. 1: Satellite image of Abu-Qir Bay showing different types of pollutions at least four different lands based point sources for pollution can be identified in Abu-Qir Bay (Moufaddal, 2005)

Sample Preparation and Analysis:

A drop of blood from caudal vessels was directly smeared on glass slides and air-dried. Smears of immature erythrocytes were prepared directly; using a small piece of cephalic kidney gently dragged along a clean slide and allowed to dry for 1–2 h (Baršiene, 1980). Smears were fixed in methanol for 10 min and were stained with 5% Giemsa solution for 8 min (Baršiene *et al.*, 2004). The frequency of micronuclei, nuclear buds, binuclei and fragmented-apoptotic cells was evaluated by scoring at 1000x magnification, using an Olympus BX51 microscope. MN were identified according to the following criteria: (1) spherical or ovoid-shaped extra nuclear bodies in the cytoplasm, (2) a diameter of 1/3 - 1/20 of the main nucleus, (3) non-refractory bodies, (4) colour, texture and optical features resembling those of the nucleus, and (5) the bodies are completely separated from the main nucleus. A total of 4000 erythrocytes from peripheral blood were examined for each fish specimen.

Micronuclei were identified according to the following criteria: round- or ovoid-shaped non-refractory particles in the cytoplasm, with optical features, structure and color resembling those of the nucleus, clearly detached from the main nucleus and with a diameter of 1/3–1/20 of the main nucleus. Other nuclear abnormalities, binucleated cells, nuclear buds (NB) and fragmented- apoptotic cells (FA), were identified using criteria described by Fenech *et al.* (2003). Nuclear buds were characterized as extruded nuclear material that appears like a micronucleus with a narrow nucleoplasmic bridge to the main nucleus. Fragmented-apoptotic cells in early stages were identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries, late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/ cytoplasmic membrane. Staining intensity in the nucleus, nuclear fragments and cytoplasm of apoptotic cells was usually greater than in viable cells.

Chromosome aberration test (CAT):

Chromosomes were prepared according to the method described by Fenocchio *et al.*, (1991) and Rita *et al.*, (2007) as follows:

(a) Small pieces of kidney and gills were extracted, and rinsed in RPMI 1640 culture medium. (b) The pieces were cut into small tissue fragments in a small plate, containing 5 ml of cooled RPMI medium (~ 4°C), and transferred the solution to a centrifuge tube, adding RPMI to a final volume of 9.5 ml. The material immersed in RPMI might be kept cool for several hours (i.e., up to 12 h) prior to performing the following steps. (c) Five drops of colchicine 0.05% (w/v) were added. (d) The solution was mixed well and the centrifuge tube was kept for 30– 35 min at room temperature (~ 20°C). (e) The material was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. (f) The hypotonic solution was added (10ml, 0.075M KCl). (g) The solution was mixed and kept for 20 min at room temperature. (h) Five drops of Carnoy's fixative methanol : acetic acid 3:1 at 4 8°C were added. (i) The material was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. (j) Fixative (6ml) was added at room temperature. (k) The material was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. (l) Steps (j) and (k) were repeated three times. (m) After the last centrifugation, the supernatant was discarded and Carnoy's fixative was added at a ratio of 1:1 (v/v) to the pellet. The solution was mixed until a homogeneous cell suspension had obtained. (n) Three drops of the cell suspension were put onto a glass slide and covered with a thin water layer at 60°C. (o) The material was stained with 5% Giemsa solution for 8 min and the slides were washed under the tap water (Rita *et al.*, 2007).

Data obtained from this investigation were statistically analyzed according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Modern civilization always creates industrialization to balance the economic conditions of the country. But the by-products and waste products of the industries may cause adverse effects on the environment. The aquatic environment is the ultimate destination for almost all industrial wastes. Water quality has become seriously impacted by these waste products. Water quality means the current status or condition of the water in a specific aquatic ecosystem. Generally each water body contains some physical and chemical properties, such as dissolved oxygen content, chloride content, and hardness, natural buffering capacity which allow the water to adopt and compensate for normal changes in the environment. Pollution occurs when conditions exceed the watershed's ability to compensate for the changes. Polluted water may be discoloured, possess a high coating on the bottom of the water body or may show no visible sign at all of pollution Belpaeme *et al.* (1997).

Aquatic organisms, such as fish, accumulate pollutants directly from the contaminated water and indirectly by feeding on contaminated aquatic organisms (Sasaki, *et al.*, 1997). The grey mullet (*Mugil cephalus*) is the most widespread among mugilid species, and inhabits the tropical and subtropical seas of the world.

In marine and freshwater ecosystems, cytogenotoxicity assays such as those evaluating micronuclei and nuclear abnormality rates have been widely employed to monitor wild areas with different levels of contamination, using a variety of organisms as marker species, ranging from mussels to fishes (Rodriguez-Cea *et al.*, 2003). Micronuclei are the result of genotoxic damage, evidenced through small masses of chromatin loose in the erythrocyte cytoplasm during mitosis, whereas nuclear abnormalities caused by cytotoxic damage are evidenced by the abnormal nuclear shape of the erythrocytes that can be characterized as blebbed, lobed, notched, vacuolated or conical, as described by Carrasco *et al.* (1990).

This work was planned to investigate the possibility of employing *Mugil cephalus* genome as a biomonitor for genetic lesion induced by aquatic contaminants. Thereby, chromosomal aberration, micronuclei, nuclear buds, fragmented-apoptotic cells and binucleate cells were investigated in fish caught from different locations along Abu-Qir Bay (Fig. 1). These locations, however display differential environmental stresses (UNEP, 1996).

Chromosomal Aberrations (CA):

Table (1) and Figs. (2 & 7) show the different types of aberrations obtained in gill and kidney cells of fish caught from the different locations. These types were stickiness, fragments, and deletions. Their percentages were proven to be higher in gills than those of kidneys. This result, however, is expected, since gill is the first target facing by aquatic contaminants. Analysis of chromosomal aberrations showed that environmental pollutants in aquatic environment promote structural alterations (e.g. gaps and breaks) but no numerical changes were observed. Unfortunately, only a small number of fish species are suited for cytogenetic investigation because of their large number of chromosomes and /or small size. Moreover, the mitotic index in fish is too low when compared to that of mammals (Cross and Hose, 1988). Chromosome aberration test have not been widely used in fish due to the characteristics of their karyotypes. In this work, it was frequently observed chromatin de-condensation which it was scored as chromosome aberrations. However, data observed from this work confirmed the levels of heavy metals detected by Aboul-Ela (2008).

Nuclear Abnormalities:

There have been a considerable number of researches that reports upon the studying clastogenicity by employing the alteration of nuclear shape. However, among current cytogenetic techniques, micronuclei and some other nuclear abnormalities are considered to be sensitive indicators for genotoxicity and cytotoxicity. The micronucleus test has been mostly applied in fish peripheral blood erythrocytes. In recent years, several studies investigating other nuclear abnormalities such as nuclear buds, or bi-nucleated cells in fish, which may be used for the assessment of genotoxicity and cytotoxicity of contaminants have appeared. Increased frequencies of nuclear abnormalities were detected in erythrocytes of the fish common minnow (*Phoxinus phoxinus*) exposed to organic contaminants (Ayllon and Garcia-Vazquez, 2000), and in the fish exposed to textile mill effluents (Cavas and Ergene-Gozukara 2003). Elevation of nuclear abnormalities was often observed in fish captured from contaminated sites (Carrasco *et al.*, 1990; Bombail *et al.* 2001). An increase in nuclear buds and micronuclei was reported in fish inhabiting zones of Goksu Delta in Turkey, which are polluted with Cu, Cd, Ni, Pb (Ergene-Gozukara *et al.*, 2007).

In this work micronucleated red cells in peripheral blood were investigated. Table (2) shows the percentages of micronucleated red cells of fish caught from the different locations that display differential environmental stresses. Micronucleus as shown in Table (2) and Fig. (3) indicate that this genotoxic bioassay is a very sensitive biomonitor for aquatic pollution. Its percentage ranged from 1.1 to 2.0 % for the tested locations 1 & 4, respectively. The number of micronuclei in fish cells is a highly variable feature and different authors have reported different numbers of spontaneous micronuclei for the same species (Gustavino *et al.*, 2001).

Regarding nuclear buds (NB), this type of abnormalities was examined in gills and kidneys (Table 3 and Fig. 4). It was noted that this type was observed in a percentage that ranged from 1.3 to 4.6% in kidney and from 1.2 to 3.3 % in gills, respectively.

Various authors (Hoofman and Raat, 1982; Ayllon and Garcia-Vazquez, 2000) have suggested that variations in the shape of nucleus could represent an alternative approach for detecting genotoxicity. In spite of the fact that a correlation between nuclear abnormalities and genotoxic effects has not yet been established, preliminary observations strongly suggest that such morphological alterations could be a manifestation of the effects of xenobiotics (Cavas *et al.*, 2005).

With respect to fragmented-apoptotic cells (FA) Table (4) illustrates the data observed from the analysis of fragmented- apoptotic (FA) cells in gills of fish caught from the different locations. Its ratio as a percentage ranged from 1.8 to 11.4 % for the locations 1 and 4, respectively. Binucleated cells (BN) are shown in Table (5) and Fig. (6), this type of aberrations observed in a percentage that ranged from 1.6 to 7.9% for in kidney and from 2.6 to 8 % in gills for the locations 1 and 4, respectively. Table (6) summarizes means and standard deviations of the different types of abnormalities in grey mullet caught from different locations. Statistical analysis revealed that the tested locations, at the level of all employed assays, are found to be significantly different from each other.

The micronucleation (MN) and binucleation (BN) testes one of the simplest, short-term tests for genetic biomonitoring in which the quality of water is known. This is a suitable and effective method to use in fishes because of its simplicity and easy scoring. Micronuclei are small, intracytoplasmic masses of chromatin resulting from chromosome breaks after clastogenic action or whole chromosomes that do not migrate during anaphase as a result of aneugenic effect (Ferraro *et al.*, 2004 and Cavas *et al.*, 2005). In case of binucleation, it is observed that the cells divide abnormally due to blocking of cytokinesis which leads to genetic imbalance in the cells and may be involved in carcinogenesis (Cavas *et al.*, 2005). The MN and BN tests have been successfully used as a measure of genotoxic stress in fish. The induction of micronuclei and bi-nucleated cells observed in fish erythrocytes, as well as in gill and kidney cells confirmed the suitability of the approach in native fish species for the screening of genotoxic compounds in aquatic ecosystems (Cavas *et al.*, 2005). The findings of the present study indicate that the piscine micronucleus test in peripheral blood is a valid technique for the assessment of genotoxic effects caused by contaminants. In addition to the MN test, induction of other nuclear abnormalities also showed signs of cytological damage.

Cytogenotoxicity analyses have been considered an efficient pollution indicator, especially in view of their sensitivity and correlation to the environmental contamination. Also, genotoxicity data can be used as early warning signs of degradation, allowing the implementation of control measures whenever a biological risk is detected (Pisoni *et al.*, 2004).

It could be concluded from this investigation that the environmental quality is probably inducing the genetic responses detected in *Mugil* sp. In a broad view, these cytogenotoxic effects may ultimately induce physiological damage and increasing levels of mutation and neoplasia in this species, leading to an ecological imbalance. Furthermore, the MN and NA frequencies of this species can be used as biomarkers of exposure to and effect of contaminants with a genotoxic potential in environmental monitoring studies.

Morphological features of chromosome aberrations, micronucleated cells, binucleated cells, nuclear buds and fragmented-apoptotic cells are shown in Figs. (7, 8a and 8b).

Table 1: Means of *chromosomal abnormalities in gills and kidneys of grey mullet caught from different locations.

Location	Stickiness		Fragments		Deletion		Total	
	G	K	G	K	G	K	G	K
1	12	8	1	1	4	3	17	12
2	6	4	2	3	5	2	13	9
3	27	22	3	2	9	4	39	28
4	41	23	5	2	11	6	57	31

*100 cells were examined.

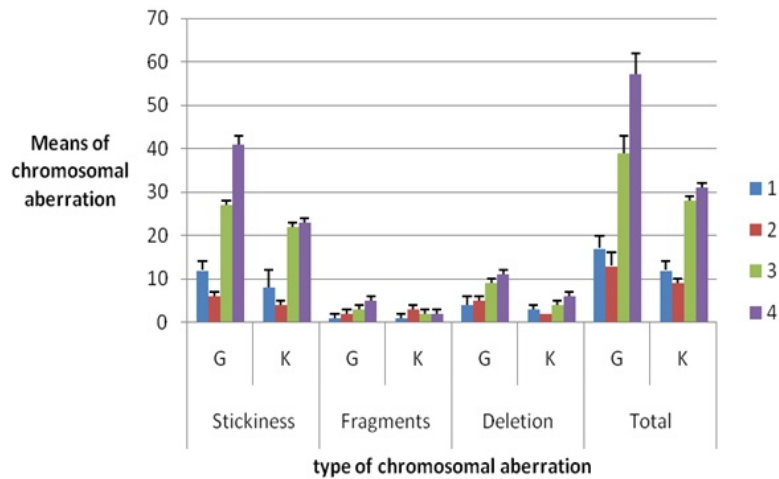


Fig. 2: Frequency of chromosomal aberrations in gills and kidneys of grey mullet caught from different locations.

Table 2: Means of micronucleated erythrocytes examined in peripheral blood in grey mullet caught from different locations.

Location,	No. red cells counted	Means of micronucleated cells	%
1	3000	53	1.100
2	3000	42	1.050
3	3000	70	1.725
4	3000	79	2.075

*: From peripheral blood.

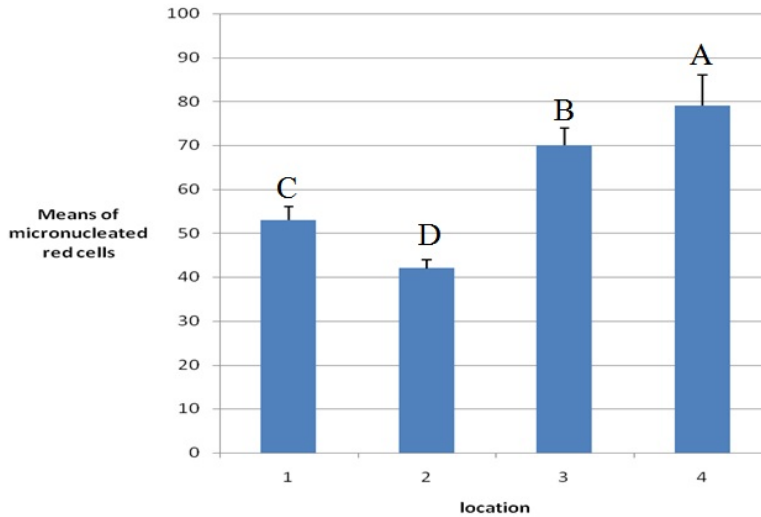


Fig. 3: Means of micronucleated erythrocytes grey mullet caught from different locations. Means with the same letter are not significantly different at significance level 0.05.

Table 3: Means of nuclear buds (NB) in gills and kidneys of grey mullet caught from different locations.

Location,	Total cells counted		Nuclear buds		%	
	G	K	G	K	G	K
1	3000	3000	36	40	1.2	1.333
2	3000	3000	55	63	1.73	2.800
3	3000	3000	70	96	2.43	3.200
4	3000	3000	114	130	3.38	4.667

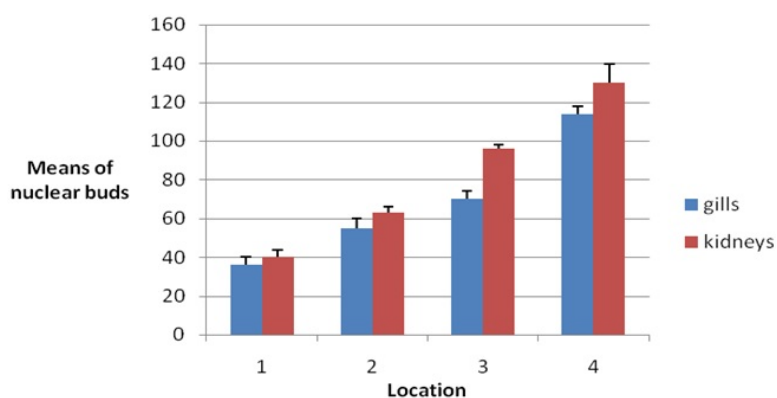


Fig. 4: Means of nuclear buds in gills and kidneys of grey mullet caught from different locations.

Table 4: Means of fragmented apoptotic cells in gills of grey mullet caught from different locations.

Location,	No. cells counted	No. apoptic cells	%
1	3000	72	1.8
2	3000	134	3.35
3	3000	238	5.95
4	3000	457	11.425

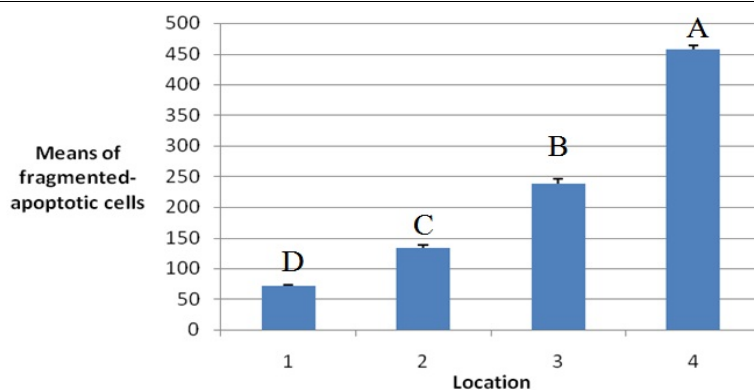


Fig. 5: Means of fragmented apoptotic cells in gills of grey mullet caught from different locations. Means with the same letter are not significantly different at significance level 0.05

Table 5: Means of binucleate cells in gill and kidney cells of grey mullet caught from the different locations.

Location,	No. examined cells		No. binucleate cells		% Binucleated cell	
	G	K	G	K	G	K
1	3000	3000	79	48	2.63	1.6
2	3000	3000	130	97	4.3	3.24
3	3000	3000	155	140	5.17	4.67
4	3000	3000	231	238	8	7.94

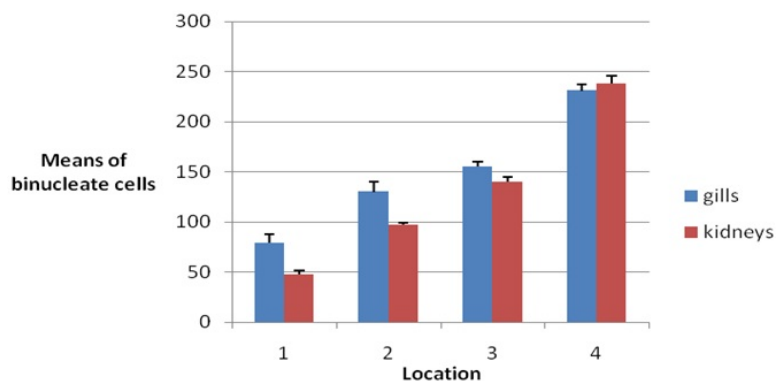


Fig. 6: Means of binucleate cells in gill and kidney of grey mullet caught from the different locations.

Table 6: Summarizes means \pm SD of the different types of abnormalities in grey mullet caught from different locations.

locations	Chromosomal aberrations(Mean \pm SD)		Nuclear alterations (Mean \pm SD)							
			MN		NB		FA		BN	
	G	K			G	K			G	K
1	17 \pm 3	12 \pm 1	53 \pm 3	36 \pm 4	40 \pm 4	72 \pm 2	79 \pm 9	48 \pm 4		
2	13 \pm 3	9 \pm 1	42 \pm 2	55 \pm 5	63 \pm 3	134 \pm 4	130 \pm 10	97 \pm 2		
3	39 \pm 4	28 \pm 1	70 \pm 4	70 \pm 4	96 \pm 2	238 \pm 8	155 \pm 5	140 \pm 3		
4	57 \pm 4	31 \pm 1	79 \pm 7	114 \pm 4	130 \pm 40	457 \pm 7	231 \pm 6	238 \pm 8		

MN: Micronuclei, NB: Nuclear buds, FA: Fragmented- apoptotic cells, BN: Binucleated cells, G: Gills and K: Kidneys.

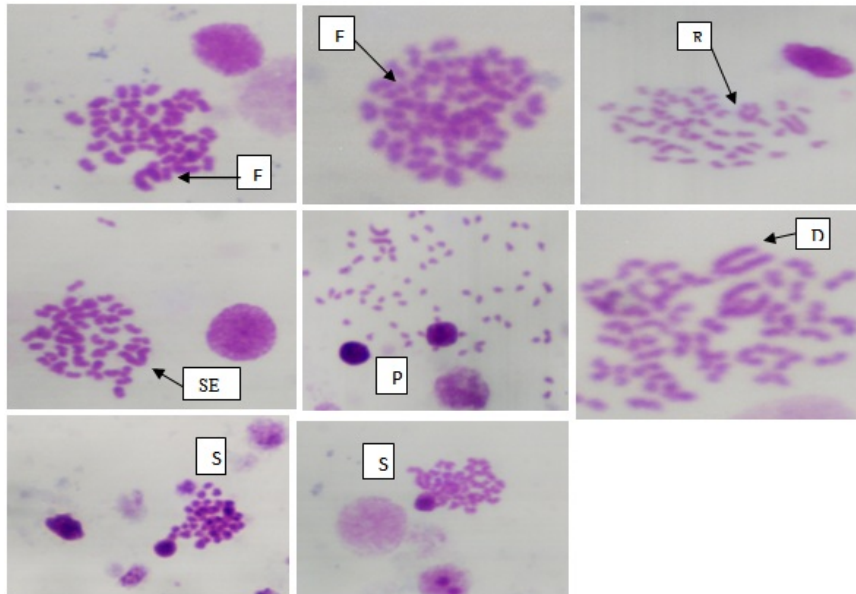


Fig. 7: Photomicrographs showing different types of chromosomal aberrations in grey mullet caught from different locations (D: Deletion, F: Fragment, S: Stickiness, R: Ring, SE: Sticky end and P: Polyploidy).

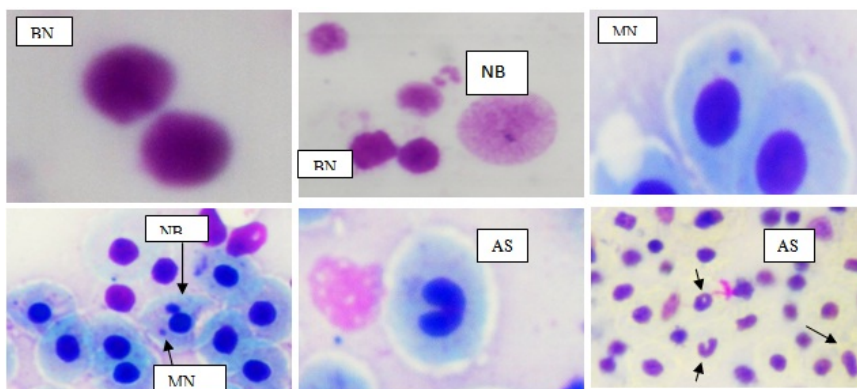


Fig. 8a: Photomicrographs showing different types of nuclear alterations in grey mullet caught from different locations (BN: Binucleated cells, NB: Nuclear buds and MN: Micronucleated cells, AS: abnormal shape of nucleus).

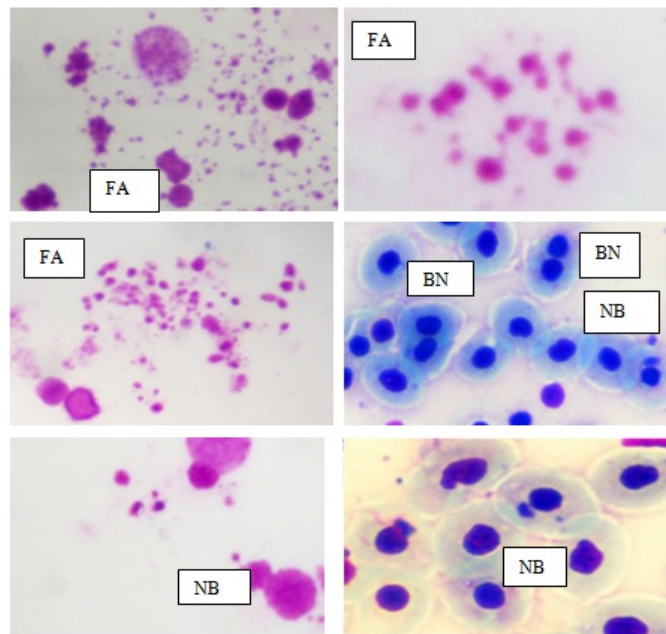


Fig. 8b: Photomicrographs showing different types of nuclear alterations in grey mullet caught from different locations (BN: Binucleated cells, NB: Nuclear buds and FA: Fragmented-apoptotic cells).

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