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BIOMARKERS IN TILAPIA (*Oreochromis aureus*) FROM THE EDKU LAKE (ALEXANDRIA) AS EVIDENCES FOR GENOTOXIC AND IMMUNOLOGICAL EFFECTS

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Aquatic animals have, often, been used in bioassays to monitor water quality (Carins *et al*., 1975; Brugs *et al*., 1977). The development of biological monitoring techniques based on fish offers the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants (Poele and Strick, 1975; Badr and El-Dib, 1978).

Lakes Waterfall is important sinks of pollutants derived from anthropogenic activities. Fish inhabiting these areas have been proposed as bioindicator for pollution monitoring through assessment of sensitive biomarkers.

Biomarkers can be defined as a change in biological response, ranging from molecular through behavioral changes, which can be related to exposure or effects of environmental contaminants (Depledge *et al*., 1995).

Biomarkers represent any biochemical and behavioral (or other) alteration that can modify the well-being of organism. Several molecular and cellular components in different fish species have been used as biomarkers for pollution exposure and effect, including biochemi-

cal, immunological and genetic parameters (Van der Oost *et al*., 2003).

Fish are excellent subject for the study of various effects of contaminants present in water samples since they can metabolize, concentrate and store water borne pollutants (Elshehawi *et al*., 2007). Biomarkers for water pollution are early diagnostic tools for biological effect measurement and environmental quality assessment (Cajaraville *et al*., 2000). Tilapia is among many fish species that are used for this proposal. They represent different sensitivity for environmental pollutants. The biological effects of pollutants were measured in Edku Lake using Tilapia *(Oreochromis aureus*) as bioindicator samples and were examined for the activities of glutathione-S-transferase acid. Liver glutamic oxaloacetic transaminases (GOT) and glutamic pyruvic transaminases (GPT) were used to assess the impact of long-term exposure to water borne cadmium (Cd) on *C. carpio*. Both showed increased activity in response to cadmium (De la Torre *et al*., 2000). The effect of lead and copper on certain biochemical parameters of the aquatic insect *Sphaerodema urinator* has also been estimated. The results showed an increase in the activity of acid phosphatase. Also the treated insects showed lower activities of GOT and GPT (Bream, 2003). GOT and GPT were employed to estimate the effect of accumulated residues of DDT, DDE, aldrin, dieldrin and delta-methrin. Higher level of GPT and GOT was found in samples with higher accumulation of pesticide residues. This possibly indicates a correlation between exposures of Pesticide and increased level of the two enzymes (Saqib *et al*., 2005).

This work was planned to investigate if aquatic pollutants present in the Edku Lake generate biological responses by comparing *Tilapia aureus* from this site with those collected in the non-polluted site of the north-west of the lake. To accomplish this objective, biomarkers for pollution exposure and effect responses were measured in tissue samples from *Tilapia aureus* collected from these two regions, in winter and summer. Polychlorinated biphenyls (PCBs) as pollutants induce effects on the immune system (Smialowicz *et al*., 1989). This study was performed to detect pollution in Lake Waterfall.

MATERIALS AND METHODS

1. Materials

Fish sampling was obtained in July 2007 (summer) and December 2007 (winter) from two sites of the Edku Lake (Alexandria, Egypt). The first site is located at the south of the lake, which was considered as a polluted site. The second site is located at the north-west of the lake as a non polluted site (Fig. 1). Fish collection was performed for 20 min using a trawl-net. Fish between 10 and 15cm were anaesthetized with benzoacine (50 ppt) and weighed. Blood samples were taken from the caudal artery with heparinized syringes. Samples were immediately used for comet assay and

micronucleus test. Liver, spleen and anterior kidney were immediately dissected. Livers were frozen in liquid nitrogen and then stored at -80°C until enzyme assays (catalase, glutathione-S-transferase and metallothionein-like proteins) determination. Spleen and anterior kidney were collected, placed in 10 ml of salt solution (HBSS: 200 mM NaCl, 1 Mm KCl, 0.2 Mm Na_2HPO_4, 0.08 Mm K_2HPO_4, 1.2 mM glucose, 100 U/ml sodium heparin,100 U/ml penicillin and 100 mg/ml streptomycin: pH 7.4) and maintained on ice until leukocyte respiratory burst measurements, as described below. Water chemistry parameters (salinity and dissolved oxygen) and temperature were measured directly at Edhu Lake.

2. Methods

2. 1 Biomarkers for pollution effect

Lipid peroxidation (LPO) was determined in liver samples according to Hermes-Lima *et al*. (1995). The method is based on the oxidation of Fe^{2+} by lipid hydroperoxide (fox reactive substances) at acidic pH in the presence of Fe^{3+}-complexing dye, xylenol orange (Sigma). Samples were homogenized (1:15 w/v) in 100% cold (4°C) methanol. The homogenate was then centrifuged at 6000 xg for 10 min at 4°C. The supernatant was collected and used for LPO determination (580 nm). Cumene hydroperoxide (CHP; Sigma) was employed as a standard.

DNA damage was assessed through two tests: comet assay that detect DNA double or single strand breaks which can be repaired; and micronucleus test (MN) that detect irreversible genetic damage. Comet assay was performed as described by Singh *et al*. (1988) and Tice *et al*. (2000) with some modifications. Fully frosted microscope slides were coated with 300 µl of 0.65% normal melting point agarose (NMPA; Gibco BRL) in TAE buffer (40 mM tris-acetate and 1 mM EDTA). Tilapia blood samples were diluted (5:1000 v/v) in phosphate buffer saline (PBS) and 20 µl of 0.65% low melting point agarose (LMPA; Gibco BRL) which prepared with Kenny's salt solution (400 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4 and 2 mM $NaHCO_3$ at pH 7.5) at 30°C (stein- ert, 1995). Slides were immersed in freshly made lysing solution (10% DMSO, 1% Triton X 100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% sodium sarcosinate at PH10) overnight at 4°C. To allow DNA to unwind, slides were placed in an electrophoresis buffer (10 N NaOH, 200 mM EDTA: pH between 12 and 13) for 15 min. Electrophoresis was carried out at 25 V and 300 mA for 20 min. After electrophoresis, slides were washed three times with neutralizing solution (0.4 M Tris at pH 7.5) and stained with 70 µl of ethidium bromide (20 µ/ml). The presence of comets was examined using a Zeiss axioplan fluorescent microscope (400 X). DNA migration was visually determined in 100 cells. Comets were classified into five different groups: 0 for intact cells: 1, 2 and 3 for intermediary levels of breaks: and 4 for maximum damage. Results were expressed as scores, where 0 represents the absence of damage and 400 indicates

the highest damage registered in the 100 analyzed cells.

Micronucleus test was performed as described by Hooftman and de Raat (1982). A drop of tilapia blood was smeared on microscope slides and air-dried. After fixation with methanol for 10 min, washed stained with 5% Giemsa (Merck) in phosphate buffer (60 mM KH_2PO_4 and 60 mM Na_2HPO at pH 6.8) for 20 min, washed with distilled water and air dried. The relative frequency of micro nucleated erythrocytes per slide was determined. Isolated leucocytes were then collected, resuspended in a 5 ml of HBSS and centrifuged at 400 xg for 10 min.

For respiratory burst measurements, head, kidney and spleen leukocytes were isolated following the procedures described by Chung and Secombes (1988). Each sample was analyzed in triplicate. The micro plate was then incubated for 30 min at 25°C in a wet chamber with occasional shaking and centrifuged at 200 xg for 10 min. Obtained pellets were re-suspended in a 250 µl of PBS after centrifugation at 200 xg for 10 min, and fixed with a 100 µl of 100% methanol for 1 min. In case well, a 150 µl of 70% methanol was added and the micro plate was centrifuged at 400 xg for 5 min. Supernatants were removed and the micro pellets were air-dried overnight at 25°C. Pellets were re-suspended in a 20 µl of 0.1% Triton X 100 solution. After 30 min, they were solubilized in a 140 ml of a 2 M KOH solution; a 120 ml of dimethyl-sulfoxide (DMSO) was added and mixed by pipette. The optical density was then measured in a spectrophotometer at 630 nm.

2.2 Biomarkers for pollution exposure

To determine catalase (CAT) and glutathione-S-transferase (GST) activities, liver samples were processed according to previously established protocols with minor modifications (Livingstone, 1988). Samples were homogenized (1:5 w/v) in cold (4°C) buffer solution containing 20 mM Tris-base, 1 mM EDTA, 1 Mm dithiothreitol (Sigma) and 500 mM of sucrose, 150 mM KCl and 0.1 mM phenylmethylsulphonyl floride (Sigma) at pH 7.6. Homogenates were then centrifuged at 9000 xg for 30 min at 4°C. The supernatant of each sample was collected and stored at -20°C.

Catalase (CAT) activity was determined following the method described by Beutler (1975), which measure the rate of enzymatic decomposition of H_2O_2 (Merck, Darmstadt, Germany) as absorbance decrements at 240 nm. Enzyme activity was expressed in CAT units, where one unit is the amount of enzyme needed to hydrolyze 1 µmol of H_2O_2/ min/mg tissue, at 30°C and pH 8.0. Glutathione-S-transferase (GST) activity was determined according the method described by Habig and Jakoby (1981). This method is based on the conjugation of 1 Mm glutathione (Sigma) with 1 mM of 1-chloro-2,4-dinitro-benzene (CDNB: Sigma), which was measured as absor-

bance increments at 340 nm and enzyme activity was expressed in GST units, where one unit is the amount of enzyme necessary to conjugate 1 µmol of CDNB/ min/mg tissue at 25°C and pH 7.0.

Metallothionein-like proteins (MT) were determined employing the method described by Viarengo *et al*. (1997). Livers were homogenized in a cold buffer solution containing sucrose (500 mM), Tris-HCl (20 Mm), PMFS (0.5 mM) and ß-mercaptoethanol (0.01%) as reducing agent at pH 8.6. MT content was estimated spectrophotometrically (412 nm) using 5.50-dithio-bis (2-nitrobezoic acid) (DTNB 0.43 mM; from Sigma). Different glutathione (GSH) concentrations ranging from 0 to 500 µM were employed as standards.

Data were analyzed through two-way ANCOVA (factors: season and sampling site; covariate: weight) followed by post-hoc mean comparisons test (normality and variance homogeneity) which were previously checked (Zar, 1984). Significance level adopted was 95%. Results were expressed as mean ± standard error.

RESULTS AND DISCUSSION

Temperature and water chemistry parameters (salinity and dissolved oxygen) were measured at the moment of fish collection as shown in Table (1). Their values were nearly similar in the two sites for both seasons. Morphometric (length and weight) parameters of collected fish are listed in Table (2). At the non-polluted site in winter, Tilapia were bigger ($P < 0.05$) than of summer ones. They were also bigger than that from the polluted site in both seasons. However, in all cases there was a parallelism in the relationship between weight and both factors (season and sampling site).

1. Biomarkers for pollution effect

1.1 DNA damage

In winter collection, Fig. (2) showed micro nucleated erythrocytes, prepared from Tilapia collected at polluted site. Both biomarkers (comet assay, CA, (Fig. 3a) and micronucleus test, MN, (Fig. 3b) showed higher ($P < 0.05$) levels of DNA damage in Tilapia from the polluted site (CA= 101.67± 7.17; MN = 0.87±0.21) than in those from the non-polluted site (CA = 38.67± 5.83; MN = 0.18±0.04). In summer, there is no significant difference ($P<0.05$) between Tilapia from the polluted site (CA = 99.4±17.65; MN = 0.32± 0.05) and the non-polluted site (CA = 57.71±11.85; MN = 0.26±0.06) although CA results showed tendency of higher values ($P<0.05$) in Tilapia from the levels of polluted site.

1.2 Lipid peroxidation (LPO) content

Tilapia collected in summer from the non-polluted site showed significantly ($P<0.05$) higher LPO content (3325.8± 438.0 nmol CHP/g tissue) than those collected in winter at the same site

(981.6±188.2 nmol CHP/g tissue). Tilapia from the polluted site showed non significant (P<0.05) difference in LPO content in summer collection (1250.5±486.4 nmol CHP/g tissue) and winter collection (1124.0±157.9 nmol CHP/g tissue), while significant difference between the two sites was only found in summer collection (Table 4), where Tilapia from the non-polluted site showed higher (P<0.05) LPO content than those from the polluted site (Fig. 3c).

1.3 Respiratory burst measurements

Nitroblue-tetrazolium reduction was higher in Tilapia from the non-polluted site than those from the polluted site. In both season collections, optical densities were high (P < 0.05) in Tilapia from the non-polluted site (winter = 0.22±0.07; summer = 0.20±0.02). No significant (P < 0.05) seasonal difference was observed in each sample site (Fig. 3d).

2. Biomarkers for pollution exposure

2.1 Enzymes (CAT and GST) activity and metallothionein-like proteins (MT) concentration

Tilapia collected in summer from the non-polluted site showed significantly (P<0.05) higher CAT and GST activities than those collected in winter at the same site (Table 3). Tilapia collected in summer from the polluted site also showed significantly (P<0.05) higher CAT and GST activities than those collected in winter at the same site (Table 3). In each season, no significant differences (P < 0.05) in CAT (Fig. 4a) and GST (Fig. 4b) activities were found between Tilapia collected from the polluted and the non-polluted sites. Also, no seasonal variations and significant differences (P<0.05) were observed in the MT concentration of Tilapia collected from the non-polluted site (winter = 2.90±0.47 and summer = 2.02 ± 0.60 µmol GSH/g of wet tissue) and the polluted site (winter = 3.13± 0.47 and summer = 2.53±0.37 µmol GSH/g of wet tissue) (Table 3).

Several field studies with different species have used the response of antioxidant and biotransformation enzymes as exposure biomarkers (van der Oost *et al*., 2003). In the present study, exposure biomarkers (CAT and GST) showed similar patterns in Tilapia from both polluted and non-polluted sites, but a clear seasonal variation was observed since values were higher in the warmer season. Water chemistry parameters also showed an important seasonal variation, being similar in both studied sites (non-polluted and polluted). In a study with the fish collected at a non-polluted site during spring and autumn, GST activity was also higher in the warmer period and CAT activity showed the same trend (Wilhelm Filho *et al*., 2001). Similar seasonal patterns of higher antioxidant and biotransformation enzymes activities in warmer seasons were also verified in other fish species (Ronisz *et al*., 1999). This response is probably related to the higher ambient temperature which can

lead to an increase in oxygen consumption and, therefore, to an enhanced reactive oxygen species (ROS) generation. Seasonal adjustments in the antioxidant defense of thermo conformers, like most of fish and invertebrates, suggested that this mechanism is a common adaptation in these species (Wilhelm Filho *et al*., 2001).

The effect of pollution on biomarkers (LPO content, DNA damage and respiratory burst measurement) showed different response patterns. LPO content in Tilapia from the non-polluted site showed a seasonal variation, being higher in summer. On the other hand, Tilapia from the polluted site did not show any seasonal variation and had lower LPO values in summer than those from the non-polluted site, a priori a non expected result. However, some authors have described the disruption of lipid metabolism due to polychlorinated biphenyls (PCBs) exposure (Ferreira and Vale, 1998). If pollutants are able to inactivate these enzymes, a possible interpretation to our results is that lipid peroxidation process could be reduced in Tilapia from the polluted site due to a lower content of polyunsaturated fatty acids (PUFAs) in the cell membranes of these fish. PCBs induced effects on the immune system that were demonstrated in several species of animals. Rats treated with Aroclor 1254 had reduced thymus weights and reduced natural killer cell activities (Smialowicz *et al.*, 1989).

In the present study, DNA damage was assessed through two tests: the comet assay (CA), which detects DNA strand breaks that can be repaired and the micronucleus test (MN), which assess mutational events. It is known that breaks detected by comet assay can be transiently presented when cells repair lesions via base or nucleotide excision. Thus, a high level of breaks in the comet assay may indicate either damage or an efficient repair process (Collins *et al*., 1997). Our results suggested that DNA damage found through comet assay in Tilapia collected in winter at the polluted site was not efficiently repaired. This statement is based on the fact that micronucleus test indicated higher levels of mutations in these fish. Some of these mutations can be related to non-repaired breaks.

The tendency of higher DNA damage values in summer collection of Tilapia did not lead to a higher micronucleus frequency, probably because breaks detected by comet assay in this season were repairing nature. It should be concluded that the DNA damage determined through CA and MN was paralleled by a lack of CAT and GST induction in fish collected at the polluted site in winter. Catalase activity is essential to promote the degradation of H_2O_2, a precursor of hydroxyl radical; a reactive oxygen species that induces DNA damage (Halliwell and Gutteridge, 1999). In this context, the lack of CAT response in fish collected in winter at the polluted site should be unable to reduce the levels of hydroxyl radical promoters. The negative correlation between anti oxidant defense

competence and DNA damage was previously verified in other aquatic organisms such as *Mytilus galloprovincialis*, where individuals with lower total antioxidant capacity also showed lower DNA integrity (Frenzilli *et al*., 2001). Several *in situ* studies have been demonstrated the occurrence of higher DNA damage in organisms collected from polluted areas, using both of the comet assay (Flamrnarion *et al*., 2002; Winter *et al*., 2004) and the micronucleus test (Minissi *et al*., 1996; Bombail *et al*., 2001), pointing to their utility in biomonitoring programs. However, a few of the cited previous studies have analyzed the response in terms of DNA damage in different seasons as an important point to be considered among the several factors that can lead to augmented damage, as registered in the present study. Further research should consider the kinetics of DNA repair to analyze the effects of pollutants on this parameter.

Results from respiratory burst assay in Tilapia collected at the two tested sites of the Edku Lake waterfall indicated that NBT reduction was higher in Tilapia from the non-polluted site than in those from the polluted site. Since previous studies reported suppression of phagocytic function by environmental contaminants (Lutz and Wasowicz, 2003), our results suggested that Tilapia phagocytes are being exposed to sub lethal concentrations of environmental contaminants in the Edku Lake waterfall, leading to manifestations of immunosuppression. These lower non-specific immune responses can lead to opportunistic diseases, such as viral infections and infestation with parasites, as previously determined in the fish Ammodytes hexapterus after hydrocarbon exposure (Moles and Wade, 2001).

Based on our results, it is not possible to point out a single chemical effect in the Edku Lake waterfall that is causing the alterations observed in the present study.

However, the researchers suggested that synergistic effects from a combination of chemicals can be affecting immune response and also causing DNA damage in Tilapia collected from the polluted site. The metabolism of several pollutants generates ROS that can attack any cellular components as DNA, fatty acids, carbohydrates and proteins, leading to serious damages to cellular macromolecules (Livingstone, 2001). Our results indicated that winter Tilapia from the polluted site were subjected to an enough level of pollutants to impair fish immunological activity and also to overwhelm the DNA repair mechanisms, generating irreversible genetic damages (mutations). However, on the contrary to the results reported by Geracitano *et al*. (2004), no significant differences in liver MT levels were found in Tilapia from the non-polluted and polluted sites. Thus, higher rates of ROS production could be deleterious for Tilapia since no responses of any important antioxidant defense,

such as catalase or GST activity, which helps to eliminate the oxidative by-products (Leaver and George, 1998), which was observed in Tilapia collected in the polluted site. Furthermore, the fact that the polluted site is characterized by higher levels of metals like copper (Seeliger and Costa, 1998; Mirlean *et al*., 2003) and hydrocarbons (Medeiros *et al*., 2005) that can generate ROS, gives support to our hypothesis.

SUMMARY

Biomarkers of exposure and effect of pollutants were analyzed in tilapia (*Oreochromis aureus*) captured in winter and summer in polluted and non-polluted sites at the Lake Edku (Alexandria, Egypt). Catalase and glutathione-S-transferase activities (exposure biomarkers) and lipid peroxidation (effect biomarker) were analyzed in liver samples. Another two effect biomarkers were also studied, blood cells DNA damage through comet assay and micronucleus test. In a broad view, results point to an important seasonal variation of the analyzed biochemical biomarkers. However, data obtained clearly indicated that tilapia collected in winter at the polluted site was subjected to a level of clastogenic agents sufficient to generate irreversible genetic damages (mutations) and impair the fish immune system.

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Table (1): Means of temperature, salinity and dissolved oxygen (Do) of Summer and Winter 2007 at the polluted and non-polluted sites of the Edku Lake water fall (North Egypt).

Site	Season	Temperature (°C)	Salinity (ppm)	Do (mg/L)
Non-polluted	Winter	13.0 ± 0.0	0.2 ± 0.1	9.2 ± 0.2
Non-polluted	Summer	24.3 ± 0.3	11.4 ± 3.8	8.5 ± 0.1
Polluted	Winter	9.5 ± 0.3	1.8 ± 0.2	10.1 ± 0.3
Polluted	Summer	24.3 ± 0.5	6.2 ± 1.7	6.7 ± 0.4

Values are expressed as means ± standard error.

Table (2): Means of morphometric data of Tilapia collected in Summer and Winter of 2007 at the polluted and non-polluted sites of the Edku Lake water fall (North Egypt).

Site	Season	Length (cm)	Weight (g)
Non-polluted	Winter	15.9 ± 0.7 a	150 ± 7.4 a
Non-polluted	Summer	13.7 ± 0.2 b	135 ± 1.3 b
Polluted	Winter	13.3 ± 0.5 b	130 ± 3.0 b
Polluted	Summer	13.9 ± 0.5 b	145 ± 1.9 b

Different letters indicate significant differences ($p<0.05$) between mean values for fish collected in the different sites and seasons.

Table (3): Enzyme activities and metallothionein-like proteins concentration in Summer 2007 and Winter 2007 at the polluted and non-polluted sites of the Edku Lake water fall (North Egypt).

Site	Season	Activity CAT (Unit)	Activity GST (Unit)	MT concentration
Non-polluted	Winter	40.0 ± 4.5	0.38 ± 0.04	2.90 ± 0.47
Non-polluted	Summer	90.8 ± 13.2	0.65 ± 0.09	2.02 ± 0.60
Polluted	Winter	40.0 ± 4.0	0.31 ± 0.04	3.13 ± 0.47
Polluted	Summer	130.0 ± 12.0	0.45 ± 0.06	2.53 ± 0.37

Table (4): Lipid peroxidation (LPO) content in Summer and Winter of 2007 at the polluted and non-polluted sites of the Edku Lake water fall (North Egypt).

Site	Season	(LPO) content (nmol)
Non-polluted	Winter	981.6±188.2 l
Non-polluted	Summer	3325.8±438.0
Polluted	Winter	1124.0±157.9
Polluted	Summer	1250.5±486.4

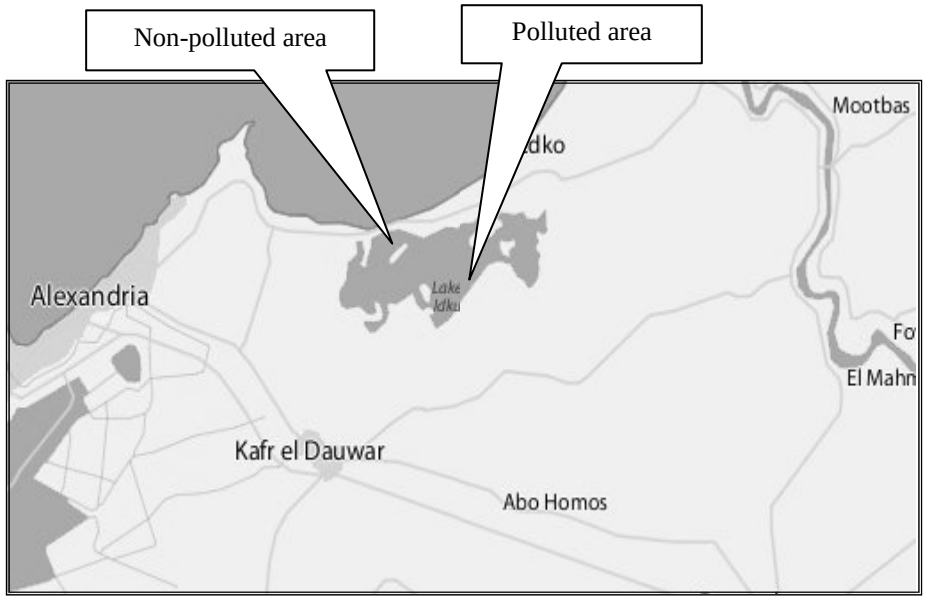

Fig. (1): Sites of Edku Lake.

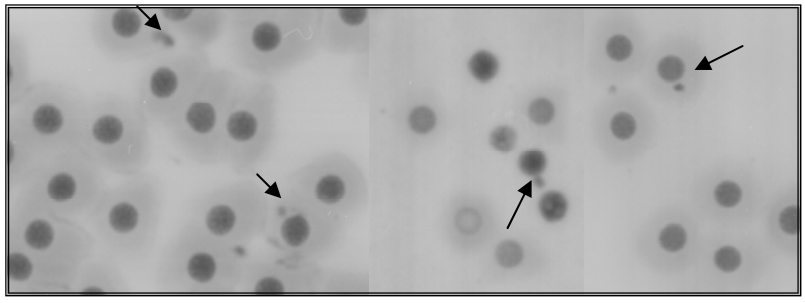

Fig. (2): The micro nucleated erythrocytes, prepared from Tilapia in polluted site. The arrows point the micronucleus beside normal nucleus.

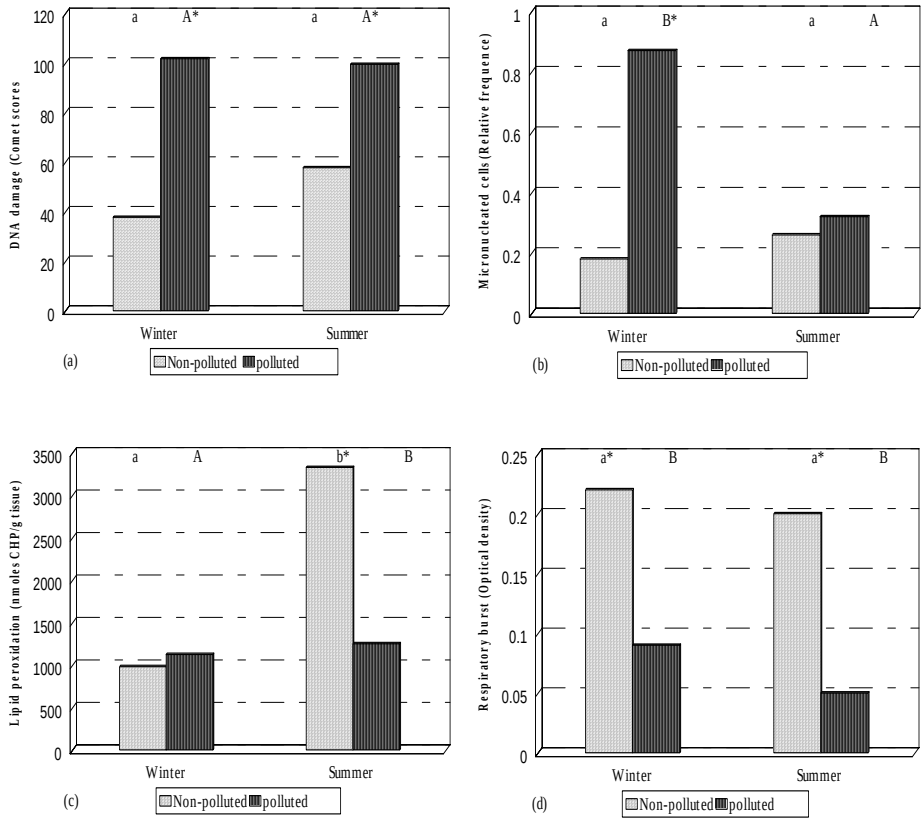

Fig. (3): Comet scores (a), relative frequency of micronucleated cells (b), Lipid peroxidation (c) respiratory burst (d), in Tilapia (*Oreochromis aureus*) collected in the non-polluted and polluted sites in winter and summer. Different letters indicate seasonal significant differences. (P<0.05) for Tilapia from the non-polluted (lower case letters) and polluted {capital letters) sites. Significant differences (P<0.05) between Tilapia from the non-polluted and the polluted site in the same season are indicated by an asterisk. Data are expressed as mean ± standard error.

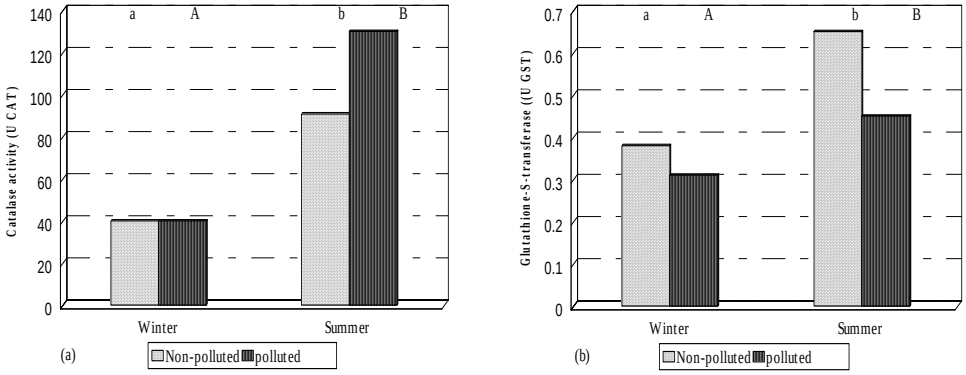

Fig. (4): Catalase (a) and glutathione-S-transferase (b) activity in Tilapia (*Oreochromis aureus*) collected at the non- polluted and polluted sites in winter and summer. Different letters indicate seasonal significant differences ($P<0.05$) for Tilapia from the non- polluted (lower case letters) and polluted (capital letters) sites. No significant differences ($P<0.05$) between Tilapia from the non- polluted and polluted site in the same season are indicated. Data are expressed as mean ± standard error.