World Journal of Fish and Marine Sciences 2 (4): 327-334, 2010
ISSN 2078-4589
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Genotoxic Effect, Biomarkers and Aquatic Contaminants in Tilapia

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Abstract: The present work was planned to investigate the relationship between aquatic contaminants and molecular biomarkers in tilapia. To achieve such a purpose tilapia fish (*tilapia zilli*) were collected from two successive seasons winter and summer in polluted and non-polluted locations at Lake Edku (Alexandria, Egypt) Catalase and glutathione S-transferase activities were estimated in liver homogenate. DNA lesion in blood cells was investigated by Comet assay. Micronucleus test was carried out in blood cells. The result obtained proved that the two tested locations displayed differential stresses and seasonal fluctuation has clearly been obtained. In addition, fish collected in winter were proven to be affected by clastogenic agents, especially at polluted location and this will affect the immune system of fish.

Key words: Biomarkers · Aquatic contaminants · DNA lesions · Tilapia · Immune system

INTRODUCTION

Aquatic animals, especially fish have often been used in bioassays to monitor and assess water quality. The development of biological monitoring techniques based on fish offers the possibility of checking water pollution with fast responses due to low concentrations of direct acting toxicants [1-3].

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 [4].

Effect biomarkers represent any biochemical, behavioral (or other) alteration that can modify the well. Being of organism. Several molecular and cellular components indifferent fish species have been used as exposure and effect biomarkers, including biochemical, immunological and genetic parameters [5].

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 [6].

Biomarkers for water pollution are early diagnostic tools for biological effect measurement and environmental quality assessment. Tilapia is among many fish species that are used for this proposed. They represent different sensitivity for Environmental pollutants.

The biological effects of water pollutants were measured in Edku Lake using Tilapia *(tilapia zilli)* as bioindicator samples were examined for the activities of glutathione -S-Transferase Acid. Liver glutamic oxaloacetic (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 [7]. 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 [8]. GOT and GPT were employed to estimate the effect of accumulated residues of DDT, DDE, aldrin, dieldrin and deltamethrin. Higher level of GPT and GOT was found in samples with higher accumulation of pesticide esidues.This possibly indicates a correlation between exposures of pesticide and increased level of the two enzymes.

This work was planned to investigate if aquatic pollutants present in the Edku Lake are generating

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biological responses by comparing *Tilapia zilli* from this site with those collected from a non-polluted site (the North-west the lake). To accomplish this objective, exposure and effect bio-marker responses were measured in tissue samples from *Tilapia zilli* collected in these two regions, in winter and summer.

MATERIALS AND METHODS

Fish sampling was performed in July 2007 (summer) and December 2007 (winter) in two sites of the Edku Lake (Alexandria, Egypt). The first one is located in a region (South the lake), which was considered the polluted site. The second site was located at the previously characterized as a non polluted site (Fig. 1). Fish collection was performed for 20 min using a trawl-net. Fish between 10 and 15cmin length were anaesthetized with benzoacine (50ppt) and weighed. Blood samples were taken from the caudal artery with heparinized syringes. Samples were immediately used for comet assay and micronucleus test, as described below. Liver, spleen and anterior Kidney were immediately dissected. Livers were frozen in liquid nitrogen and then stored at -80°C until enzyme assays and metallothionein-like proteins determination, as described below. As Spleen and anterior kidney were collected, placed in 10 mL of salt solution (HBSS:200mM NaCl, 1Mm KCl,0.2Mm Na□HPO□, 0.08Mm KH□PO□, 1.2mM Glucose, 100U/mL Sodium Heparin,100U/mL Penicillin and 100mg/mL Streptomycin: pH 7.4) and maintained on ice until leukocyte respiratory burst measurements, as described below. Water chemistry parameters (pH and temperature) were measured directly in the field.

Exposure Biomarkers: To determine catalase (CAT) and glutathione-S-trans-ferase (GST) activates, liver samples were processed according to previously established protocols with minor modifications [9]. They were homogenized (1:5w/v) in cold (4°C) buffer solution containing 20 mM Tris-Base, 1 mM EDTA, 1 Mm dithiothreitol (Sigma), 500 mM sucrose, 150 mM KCl, 0.1 mM phenylmethylsulphonyl floride (Sigma), with pH adjusted to 7.6. Homogenates were then centrifuged at 9000xg, for 30 min at 4°C. The supernatant of each sample was collected and stored at -20°C. Catalase (CAT) activity was determined [14], which measure the rate of enzymatic decomposition of H^2O□ (Merck, Darmstadt, Germany) as absorbance decrements at 240nm. Enzyme activity was expressed in CAT units, where one unit is the amount of enzyme needed to hydrolyze 1 μmol of H2O2/min/mg tissue, at 30°C and pH 8.0. Glutathione S-transferase (GST) activity was determined [15]. This method is based on the conjugation of 1Mm glutathione (sigma) with 1mM of 1-chloro-2,4-dinitro-benzene (CDNB:Sigma), which was measured as absorbance increments at 340 nm and enzyme activity was expressed in GST units, whereas 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 [16]. Livers were homogenized in a cold buffer solution containing sucrose (500mM), Tris-HCl (20Mm), PMFS (0.5mM) and ß-mercaptoethanol (0.01%) as reducing agent. The pH was adjusted to 8.6. MT content was estimated spectrophotometrically (412nm) using 5,50-dithio-bis (2-nitrobezoic acid)(DTNB 0.43mM: from Sigma).

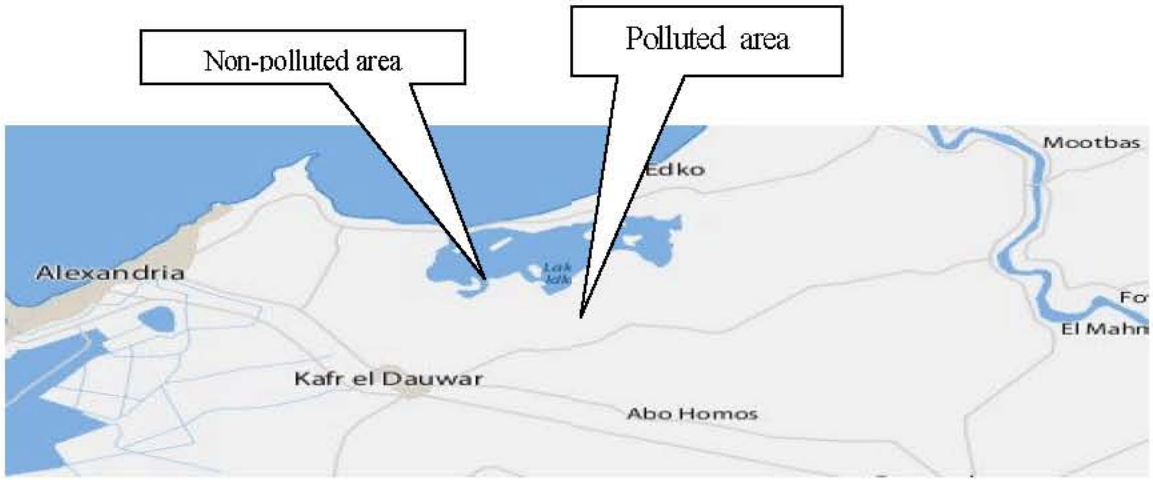

Fig. 1: Site of Edku

Different glutathione (GSH) concentrations ranging from 0 to 500μM were employed as standards.

Effect Biomarkers: Lipid peroxidation (LPO) was determined in liver samples [10]. The method is based on the oxidation of Fe^{2}□ by lipid hydroperoxides (fox reactive substances)at acid pH in the presence of the F^{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 x g, for 10min at 4°C. The supernatant was collected and used for LPO determination (580 nm). Cumene hydro peroxide (CHP; sigma) was employed as standard.

DNA Damage Was Assessed Through Two Tests: Comet assay that detect DNA double or signal strand breaks which can be repaired; and micronucleus test that detect irreversible genetic damage. Comet assay was performed [11,12] with some modifications. Fully frosted microscope slides were coated with 300μL 0.65% normal melting point agarose (NMPA; Gibco BRL) IN TAE buffer (40mM tris-acetate, 1mM EDTA). Tilapia blood samples were diluted (5:1000v/v) in phosphate buffer saline (PBS) and 20μL of 0.65% low melting point agarose (LMPA; Gibco BRL) prepared with Kenny's salt solution (400mM NaCl, 9mM Kcl, 0.7mM K□HPO□, 2mM NaHCO□, pH 7.5) at 30°C [20]. Slides were immersed in freshly made lysing solution (10% DMSO, 1% Triton X-100, 2.5M NaCl, 100mM EDTA, 10mM Tris, 1% sodium sarcosinate; pH10) overnight at 4°C. To allow DNA to unwind, slides were placed in an electrophoresis buffer (10N NaOH, 200mM EDTA: pH between 12 and 13) for 15 min. Electrophoresis was carried out at 25 V and 300mA for 20 min. After electrophoresis, slides were washed three times with neutralizing solution (0.4M Tris, pH7.5) and stained with 70μL of ethidium bromide (20μ/mL). The presence of comets was examined using a Zeiss axioplan fluorescent microscope (400X). DNA migration was visually determined in 100 cells. Comets was classified into five different groups: 0 for intact cells: 1, 2 and 3 for intermediary levels of breaks: and 4 maximum damage. Results were expressed as scores, where 0 represents absence of damage and 400 indicates the highest damage registered in the 100 cells analyzed.

Micronucleus test was performed [13]. 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 (60mM KH□ PO□ and 60mM Na□HPO:pH6.8) for 20 min, washed with distilled water and air –dried. The relative frequency of micronucleated erythrocytes per slide.

Isolated leucocytes were then collected, resuspended in 5 mL of HBSS and centrifuged at 400xg for 10 min.

For respiratory burst measurements, head-kidney and spleen leukocytes were isolated f [14].

Each sample was analyzed in triplicate. The microplate was then incubated for 30 min at 25°C in a wet chamber with occasional shaking and centrifuged at 200xg for 10 min. Pellets obtained were resuspended in 250μL of PBS after centrifugation at 200 X g for 10 min and fixed with 100 μL of 100% methanol for 1 min. In case well, 150 μL of 70% methanol were added and the micro plate was centrifuged at 400 X g for 5 min. supernatants were removed and the microplate was air-dried overnight at 25°C. Pellets were re-suspended in 20 μL of 0.1% Triton X 100 solution. After 30 min, they were solubilized in 140 mL of a 2 M KOH solution; 120 mL of dimethyl-sulfoxide (DMSO) were added and mixed by pipetting. The optical density was then measured in a spectrophotometer at 630 nm.

Exposure Biomarkers: 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) were previously checked [15]. Significance level adopted was 95%. Results were expressed as mean ± standard error.

RESULTS

Water physicochemical parameters (temperature, pH and dissolved oxygen) measured at the moment of fish collection are shown in Table 1. Values for all parameters measured were similar in the two sites for both seasons and had no relationship with any biomarker data. Morphometric (length and weight) parameters of collected fish are listed in Table 2. At the non-polluted site, winter Tilapia were bigger ($P < 0.05$) than summer ones. They were also bigger than that from the polluted site in both seasons. However, in all cases weight (covariate) did not influence the statistical analysis, being verified a parallelism in the relationship between weight and both factors (season and sampling site).

Exposure Biomarkers

Enzyme (CAT and GST) Activities and Metallothionein-Like Proteins (MT) Concentration: Summer Tilapia from the non-polluted site showed significantly ($P < 0.05$) higher CAT (90.8 ± 13.2 U CAT) and GST (0.65 ± 0.09 U GST) activities than those collected in winter at the same site (CAT = 40.0 ± 4.5 U CAT; GST = 0.38 ± 0.04 U GST).

Table 1: Environmental parameters registered in summer 2007 and winter 2007 at the polluted and non-polluted sites of the Edku lake water fall (north Egypt)

Site	Season	Temperature (°C)	Salinity	Do (mg/L)	*n*
Non-polluted	Winter	13.0 ± 0.0	0.2 ± 0.1	9.2 ± 0.2	4
Non-polluted	Summer	24.3 ± 0.3	11.4 ± 3.8	8.5 ± 0.1	4
Polluted	Winter	9.5 ± 0.3	1.8 ± 0.2	10.1 ± 0.3	4
Polluted	Summer	24.3 ± 0.5	6.2 ± 1.7	6.7 ± 0.4	4

Do: dissolved oxygen; n: number of measurements. Values are expressed as means ± 1 standard error

Table 2: Morphometric data of Tilapia collected in summer 2007 and winter 2007 at the polluted and non-polluted sites of the Edku lake water fall (north Egypt)

Site	Season	Length (cm)	Weight (g)	N
Non-polluted	Winter	15.9 ± 0.7 a	150 ± 7.4 a	10
Non-polluted	Summer	13.7 ± 0.2 b	135 ± 1.3 b	10
Polluted	Winter	13.3 ± 0.5 b	130 ± 3.0 b	10
Polluted	Summer	13.9 ± 0.5 b	145 ± 1.9 b	10

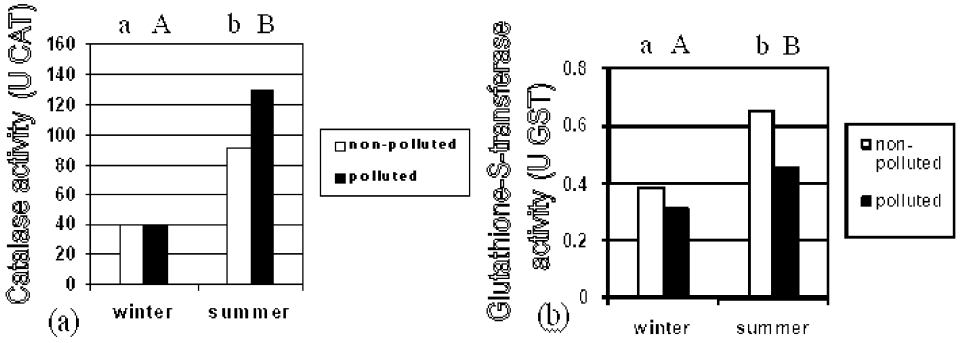

Fig. 2: Catalase (a) and glutathione-S-transferase (b) activity in Tilapia (*Oreochromis zilli*) collected at the non- polluted and polluted sites in winter and summer. Different letters indicate seasonal significant differences ($P < 0.05$) for Tilapia from the non- polluted (lower case letters) and polluted (capital letters) sites. Significant differences ($P < 0.05$) between Tilapia from the non- polluted and polluted site in the same season are indicated by an asterisk. Data are expressed as mean +1 standard error

Summer Tilapia from the polluted site also showed significantly ($P < 0.05$) higher CAT (130.0 ± 12.0 U CAT) and GST (0.45 ± 0.06 U GST) activities than those collected in winter at the same site (CAT = 40.0 ± 4.0 U CAT; GST = 0.31 ± 0.04 U GST). In each season, no significant differences in CAT (Fig. 2a) and GST (Fig. 2b) activities were found between Tilapia from the polluted and the non-polluted site. Also, no seasonal variations and significant differences ($P < 0.05$) were observed in the MT concentration of Tilapia 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).

Effect Biomarkers

Lipid Peroxidation (LPO) Content: Summer Tilapia 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 no significant (difference in LPO content in summer (1250.5 ± 486.4 nmol CHP/g tissue) and winter (1124.0 ± 157.9 nmol CHP/g tissue) significant difference between the two sites was only found in summer, when Tilapia from the non-polluted site showed higher ($P < 0.05$) LPO content than those from the polluted site (Fig. 3a).

Respiratory Burst Measurements: Nitroblue tetrazolium reduction was higher in Tilapia from the non-polluted site than those from the polluted site. In both seasons, optical density was higher ($P < 0.05$) in Tilapia from the non-polluted site (winter = 0.22 ± 0.07; summer = 0.20 ± 0.02). No significant seasonal difference was observed in each sample site (Fig. 3b).

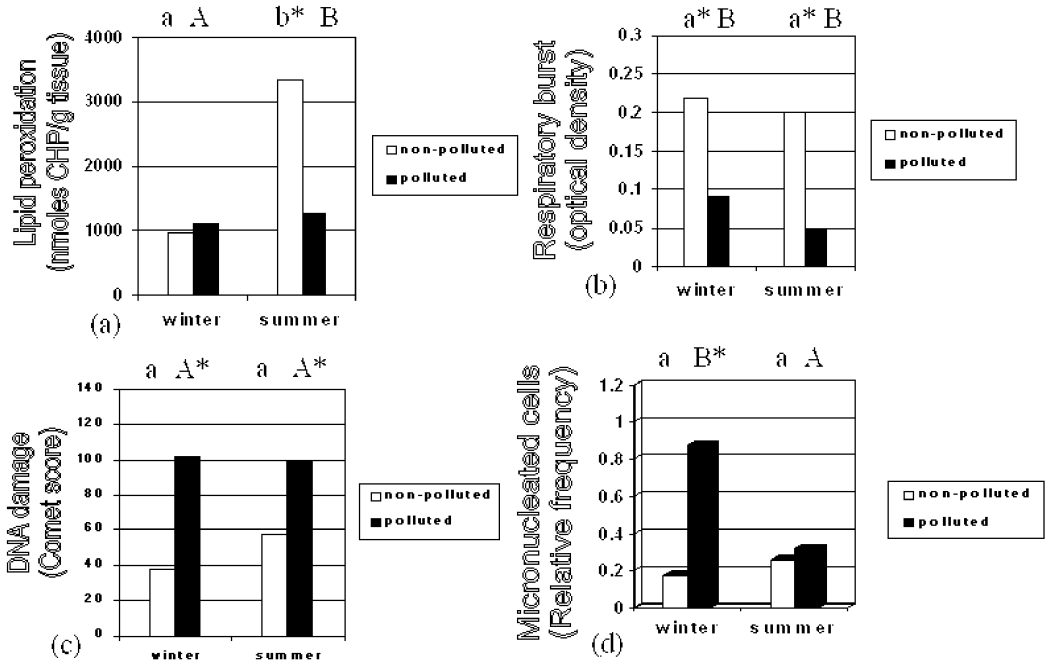

Fig. 3: Lipid peroxidation (a), respiratory burst (b), comet scores (c) relative frequency of micronucleated cells, (d) in Tilapia (*Oreochromis zilli*) collected in the non-polluted and polluted sites in winter and summer. Different letters indicate seasonal significant differences ($P < 0.05$) for 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 + I standard error

DNA Damage: In winter, both biomarkers (comet assay, CA, Fig. 3c; and micronucleus test, MN, Fig. 3d) 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 = 83.67 ± 5.83; MN = 0.18 ± 0.04). In summer, there is no significant difference 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 ($P < 0.052$) in Tilapia from the levels polluted site.

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

DISCUSSION

Several field studies with different species have used the response of antioxidant and biotransformation enzymes as exposure biomarkers [16]. In the present study, exposure biomarkers (CAT and GST) showed similar patterns in Tilapia from both polluted and no-polluted sites, but a clear seasonal variation was observed. 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 [17]. Similar seasonal patterns of higher antioxidant and biotransformation enzymes activities in warmer seasons were also verified in other fish species [16]. 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, suggest that this mechanism is a common adaptation in these species [18].

The effect 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 PCBs exposure [19]. 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 PUFAs in the cell membranes of these fish.

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 present 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 [20]. Our results suggest 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 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 stressed 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 H2O2, a precursor of hydroxyl radical; a reactive oxygen species that induces DNA damage [21]. 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 [22]. Several in situ studies have been demonstrated the occurrence of higher DNA damage in organisms collected from polluted areas, both using the comet assay [23, 24] and the micronucleus test [25, 26], pointing to their utility in biomonitoring programs However, few of the cited previous studies have analyzed the response in terms of DNA damage in different seasons 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 sites of the Edku lake water fall indicate 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 [27], our results suggest that Tilapia phagocytes are being exposed to sublethal concentrations of environmental contaminants in the Edku lake water fall, leading to manifestations of immunosuppressant. 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 [28].

Based on results presented here, it is not possible to point out a single chemical in the Edku lake water fall that is causing the alterations observed in the present study. However, synergistic effects from a combination of chemicals can be affecting immune response and also causing DNA damage in Tilapia collected at 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 [29]. Data showed here indicate that winter Tilapia from the polluted site were subjected to a level of pollutants enough to impair fish immunological activity and also to overwhelm the DNA repair mechanisms, generating irreversible genetic damages (mutations). However, contrary to results reported by Moles and Wade [30], 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 [31], 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 [32, 33] and hydrocarbons [34], that can generate ROS, gives support to our hypothesis.

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