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Histochemical and Biochemical Changes in Liver of *Tilapia zilii* G. as a Consequence of Water Pollution

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Abstract: Chemical analysis of surface water samples collected randomly from three locations in lake Maryût indicated a considerable rise of certain heavy metals and bad water quality in polluted locations (Moharrem Bey and Merghim basin), with elevated chemical and biological oxygen demands (BOD) as compared to reference area (Fish farm) characterized with lesser metal content, water turbidity and alkalinity. Cytochemical changes in glycogen, proteins and lipids content in liver of Tilapians caught from these locations were investigated. Hepatocellular accumulation of protein and lipid inclusions in association with depletion of glycogen appeared in the polluted specimens. In parallel, enhanced levels of total proteins, total lipids, cholesterol, glucose and creatinine were estimated in blood of fish inhabiting the polluted areas. It can be concluded that biochemical parameters that fluctuated in response to deleterious effects of pollution could be ranked as possible biomarkers of pollution. This study also underlines the importance of morphological studies as a link between biochemical-physiological and ecological investigations.

Key words: Lake Maryût, *Tilapia zilii*, water chemistry, histochemistry

Introduction

Water pollution is recognized globally as a potential threat to both human and other animal populations which interact with the aquatic environments (Biney *et al.*, 1987; Svensson *et al.*, 1995). According to Jqrgensen and Johnsen (1989), heavy metals (Cr, Pb, Cu, Zn, ect.) are considered as the main causes of pollution in aquatic ecosystem and are expected to be so in the future, having the highest environmental stress index, often in excess of the recommend threshold limit values. The dangers involved from the presence of heavy metals in the environment derive not only from their persistence and toxicity, but also from the remarkable degree of bioaccumulation they undergo through the trophic chain, thus becoming serious danger to man (Hernandez-Hernandez *et al.*, 1990; Bishop, 2000). Initial experimental studies with heavy metals indicated that they could impair the respiratory function of the gills, consequently death in fish is probably caused by tissue hypoxia (Skidmore, 1970; Playle *et al.*, 1993).

The conventual laboratory toxicity test, in which conditions may be controlled within desired limits, is valuable for studies of the properties of specific toxicants. However, such manipulative studies can not be extrapolated to the natural environment because they lack ecological realism (Benson and Black, 1990). Variability and interaction of environmental factors in natural habitats complicate the responses of organisms to contaminants (Adams *et al.*, 1992,1996). They also stated that the need for reliable methods to assess the impact of chemical pollution on aquatic habitats has generated an increasing interest in biochemical indicators of contaminant exposure and their effects in fish.

Hepatic protein biosynthesis and serum proteins are called upon to provide an early warning of toxicity (Casarett and Doull, 1975). Hilmy *et al.* (1987) found an increase in protein content of serum and liver in fish following cadmium and zinc toxicity.

Adequate energy (lipid) reserves are required by organisms to mediate the effects of stress (Lee *et al.*, 1983) and to serve as energy buffers during periods of harsh environmental conditions and food shortages (Adams and Mclean, 1985). These considerations indicate that a lipid compartment is important and appears necessary in any individual model that is utilized to represent the biological-chemical interaction (Hallam *et al.*, 1988). The disturbance and mode of occurrence of glycogen in the liver cells of experimental animals was reported under the influence of hydrocarbons and chemical carcinogens (Svoboda and Higginson, 1968). Elevated levels of creatinine in serum were reported in *T. zilii* inhabiting polluted locations in Lake Maryût where dissolved oxygen was depleted as well as in experimentally asphyxiation fish (Saad *et al.*, 1973). Everall *et al.* (1991) indicated similar change in adult captive Atlantic salmon exposing River Don water containing field drain discharges *in situ*. Since two decades, Lake Maryût (Alexandria, Egypt) has been vastly affected by the disposal of industrial effluents from some factories in Alexandria. This study aimed at investigating the effect of pollution in Lake Maryût on essential metabolites in liver and blood of *T. zilii*.

Materials and Methods

Study area: Lake Maryût (Fig. 1) is a small shallow lake that is situated at the southern edge of Alexandria. It has a total area of about 5500 hectares with an average water depth of 120 cm. The lake is divided artificially into:

- The lake proper represents the main basin and receives most of its water from the polluted water of Qualaa drain. Other sources of pollution include industrial waste effluents, discharged at the northeastern corner, Gheit El-Enab. Drain receiving sewage from Karmooz and El-Kabbariu outfall that discharges raw sewage at the northern West Side.
- The Fish farm, receives most of its water from Maryût, El-Gedida hydraulic pumps and the Omoum drain and connected to the Qualaa drain through a movable gate. Thus, it is not subjected to direct discharges.
- The southwest (S.W.) and southeast (S.E.) basins are totally separated from the lake by a dyke bordering the Omoum drain. The S.E. basin is nearly free of pollution while the S.W. basin is partially contaminated with mineral oils discharged by the cooling of pipes of El-Nasr Petroleum Company.

Animal used: Random fish samples of adult *Tilapia zilii* G. were collected in close meshed nets from the Fish farm (location 1), Moharrem Bey area (location 2) and Merghim basin (location 3). Fish were then transferred into large vessels filled up with aerated lake water. The sampling period lasted from December 1997 to February 1998. From each location about 100 fish of both sexes were taken at random. Total fish length and weight ranged between 9-13 cm and 17-26g, respectively. Spontaneously with fish capture, sampling of water was carried out from the particular catch area of the lake at monthly intervals. Water samples (2-3 litters) were taken about 20 cm below the water surface to avoid floating matter.

Chemical analysis of water: Water samples were analyzed for measurement of certain heavy metals and physicochemical characteristics. Standard methods for the analysis of natural and treated wastewater were undertaken according to the American Public Health Association (1975).

For histochemical evaluation: Fish from all experimental areas were dissected, the abdominal cavity was operated and the liver was

excised quickly and was fixed in 10% neutral formalin. After fixation, the specimens were processed as usual in the recognized method of dehydration cleared in xylene and finally embedded in paraffin wax. Mercury bromophenol blue (Hg-BPB) were used for total proteins Sudan black B for lipids and Best's Carmine for glycogen (Pearse, 1968).

For biochemical evaluation: Blood samples were collected from unanesthetized fish by cardiac puncture with a 1.4x 50-mm^2 non-heparinized injection needle (Lehmann and Sturenberg, 1974). Then, blood ran freely into a vial and was allowed to coagulate in frozen ice for few minutes. Samples were, eventually centrifuged for 15 min at 1000 Xg. Packed cells were discarded and the supernatant serum was decanted and stored at -20 °C. Colorimetric determinations of some non-cellular components of blood were performed using DMS 200 spectrophotometer. The absorbency was detected at an appropriate wavelength ranging form 500 to 550 nm according to the followings parameter tested:

- Total serum protein (TSP) was determined using Boehringer Mannheim GmbH protein assay kit (Hitachi 706 D/712, USA) following the Biuret reaction (Weichselbaum, 1946).
- Total lipid concentration was investigated using kits made by BioMerieux (Marcy l' Etoile/69260 charbonnieres les Basins, France) according to the method originally devised by Schmit (1964).
- Boehringer Mannheim GmbH diagnostic kits were also used to detect the concentrations of cholesterol, glucose and creatinine after Kattermann *et al.* (1984), Trinder (1969) and Seelig and Wust (1969), respectively.
- Experimental design, analysis of variance and analysis of mean differences were carried out according to Campbell (1989).

Results

Water analysis shows that locations 2 and 3 have the highest values of heavy metals. Table 1 the pH values was generally on the alkaline side in the three locations. Samples of location 2, revealed relatively high turbidity, alkalinity, COD and BOD, a great richness in phosphate, ammonia and nitrite. The DO was much depleted in water samples from location 3. Water samples from locations 2 and 3 shows a decrease in chlorosity and hardness as compared to location 1. Water samples from the Fish farm contained also considerable nuclear boundaries have exhibited intense protein stainability. In addition, it was noted that hepatic cells of the same area might contain different amounts of total amounts of salts especially phosphate and nitrate, while Merghim basin had the least concentrations (Table 1) (Adham *et al.* 1997).

Cytochemical observations

Proteins: Liver specimens of *T. zilii* caught from the Fish farm were characterized by a high concentration of total proteins. In the cytoplasm, the Hg-BPB reaction was either in the form of bluish granules of different sizes, or in a diffused state, perinuclear or peripheral in position and particularly concentrated adjacent to blood sinusoids. In the nucleus, the chromatic granules and the nucleolus were intensely stained while the nucleoplasm was stained to a lesser extent and gave a diffuse faint blue color (Fig. 2a). Also, an increase in the protein content was observed in liver tissues of fish collected from Moharrem Bey area, where the cytoplasm gave a more intense reaction than the nuclear structures (Fig. 2b). The Hg-BPB positive reaction for proteins appeared in the cytoplasm either in the form of accumulated granules particularly at the sinusoidal peripheries where plasma proteins are discharged from hepatocytes to capillaries, or vice versa, or as a sort of coagulation in a diffused state. It is worthy to mention that total proteins acquired a normal appearance in some cells.

Merghim basin specimens revealed a dramatic increase of total proteins in most liver tissues (Fig. 2c). This increase was in both number and size of the positively stained granules. As described in reference tissues, adjacent areas and even adjacent cells showed variability in the content of protein.

Lipids: In liver specimens of *T. zilii* caught from the Fish farm, lipoid inclusions were uniformly distributed throughout the cytoplasm, perinuclear and peripheral in position, and particularly accumulated in the cytoplasm adjacent to sinusoids (Fig. 3a). The nuclei were mostly sudanophobic. Also, it was observed that in these preparations that the individual hepatocytes have designated a variant trend of lipid localization, some cells appeared more condensed with lipid than other ones.

The fat content of liver tissues of fish collected from Moharrem Bey area was appeared to be much more abundant than the reference specimens. The sudanophilic granules were coarser and showed a tendency to aggregate into patches either surrounding the nucleus or lying at the periphery of the cells. Most hepatocytes exhibited extremely strong diffuse patterns of Sudan black stainability. However, the liver cells showed markedly detectable alterations in the quantity of lipids, some of them displayed a reduced Sudan black reactivity. It is worthy to mention that the two patterns of cells were distributed in non-consistent manner, they were randomly intermingled with each other. The nuclei appeared sudanophilic (Fig. 3b).

A drastic lipid augmentation could be noted in the hepatic tissues of *T. zilii* collected from Merghim basin (Fig. 3c). Some hepatocytes were manifesting considerable rise of sudanophilia even comparable to specimens from Moharrem Bey area. Lipoid inclusions were frequently confined to the intervacuolar as well as the marginal cytoplasmic areas of these cells.

Glycogen: On the other hand, it was found that in liver specimens of *T. zilii* caught from the Fish farm that glycogen granules were distributed more or less evenly throughout the cytoplasm, but in the majority of cells glycogen was aggregated into clumps (Fig. 4a). Such polarization is commonly termed "glycogen flight". Hepatic tissues of fish collected from Moharrem Bey area showed a decrease in glycogen content as well as heterogeneous picture of glycogen distribution (Fig. 4b). Accordingly, some hepatocytes were still rich in these inclusions but in concomitant with regression of the phenomenon of glycogen flight, others had lost a considerable proportion of these materials and the rest had undergone a rather complete deprivation of glycogen. These features of glycogen disturbances encountered in Moharrem Bey specimens were obviously more exaggerated in those of Merghim basin where conspicuous depletion of glycogen inclusions was observed (Fig. 4c). In many cells, glycogen was less prominent or absent.

Biochemical Observations: Result of the quantitative determination of serum total protein, total lipids, cholesterol, glucose and creatinine as well as statistical analysis of mean differences (multiple range tests) are presented in Table 2 (Adham *et al.* 1997). Serum total protein showed a wide spectrum of mean values as 4.15 g/dl in the fish form (less polluted or reference area), 5.38 g/dl in Moharrem Bey area (moderately polluted) and 6.61 g/dl in Merghim basin (heavily polluted). TSP revealed a significant rise in the mean difference between Merghim basin and the Fish farm. Mean differences between the remaining locations (Moharrem Bey and the Fish farm and Merghim basin and Moharrem Bey) were found to be insignificant.

All mean differences of total lipids, cholesterol, glucose and creatinine were significant. Fish caught form Moharrem Bey and Merghim basin showed a state of hyperglycaemia and lipidaemia. Elevated levels of creatinine in serum were also reported in these locations as compared to the Fish farm (less polluted). The mean values increased in the following rank:

Merghim basin > Moharrem Bey > Fish farm.

Table 1: Levels of heavy metals and physicochemical parameters in water samples collected from locations in Lake Maryût (Adham *et al*., 1997

	Locations		
Heavy metals (mg/l)	Fish farm	Moharrem Bey	Merghim basin
Iron	0.740	20.50	16.420
Cadmium	0.008	0.09	0.083
Chromium	0.109	0.47	0.660
Lead	0.030	0.23	0.753
Copper	0.227	0.94	0.639
Manganese	0.016	0.06	0.042
Zinc	0.218	0.41	0.516
Physicochemical parameters			
pH	8.20	7.90	8.45
Turbidity (NTU)	14.00	32.00	6.00
Dissolved oxygen (mg/l)	7.34	6.60	4.00
Chemical oxygen demand (mg/l)	123.00	150.00	100.00
Biological oxygen demand (mg/l)	65.00	95.00	75.00
Hardness (mg/l)	700.00	420.00	480.00
Alkalinity (mg/l)	390.00	450.00	360.00
Chlorosity (mg/l)	3100.00	2500.00	2600.00
Nutrient salts (mg/l):	0.58	2.47	0.31
PO_4-P	0.16	0.43	0.10
NH_4-N	3.75	4.00	1.50
NO_3-N	0.08	0.10	0.06
NO_2-N			

Each value represents the average of three experiments. NTU : Normal turbidity unit

Table 2: Biochemical evaluation of some blood components of *Tilapia zilii* collected from three locations (Adham *et al*., 1997)

Locations	(Fish farm)	(Moharrem Bey)	(Merghim basin)
Total proteins (g/dl)	4.15 ± 0.25^{a}(10)	5.38 ± 0.24^{ab} (10)	6.61 ± 0.64^{b} (10)
Total lipids (mg/dl)	370.60 ± 12.26^{a} (10)	448.21 ± 17.30^{b} (11)	599.55 ± 16.86^{c}(10)
Cholesterol (mg/dl)	112.76 ± 4.24^{a} (10)	165.62 ± 7.05^{b} (9)	201.13 ± 9.72^{c} (9)
Glucose (mg/dl)	81.29 ± 1.79^{a} (8)	101.71 ± 2.77^{b}(11)	112.88 ± 2.61^{c} (11)
Creatinine (mg/dl)	1.75 ± 0.14^{a} (12)	2.53 ± 0.22^{b} (10)	3.24 ± 0.28^{c} (11)

Values are presented as means ± S.E., similar superscripts (a, b & c) differ insignificantly, different superscripts differ significantly ($\alpha = 0.05$). Number of samples employed in each assay are included between parentheses.

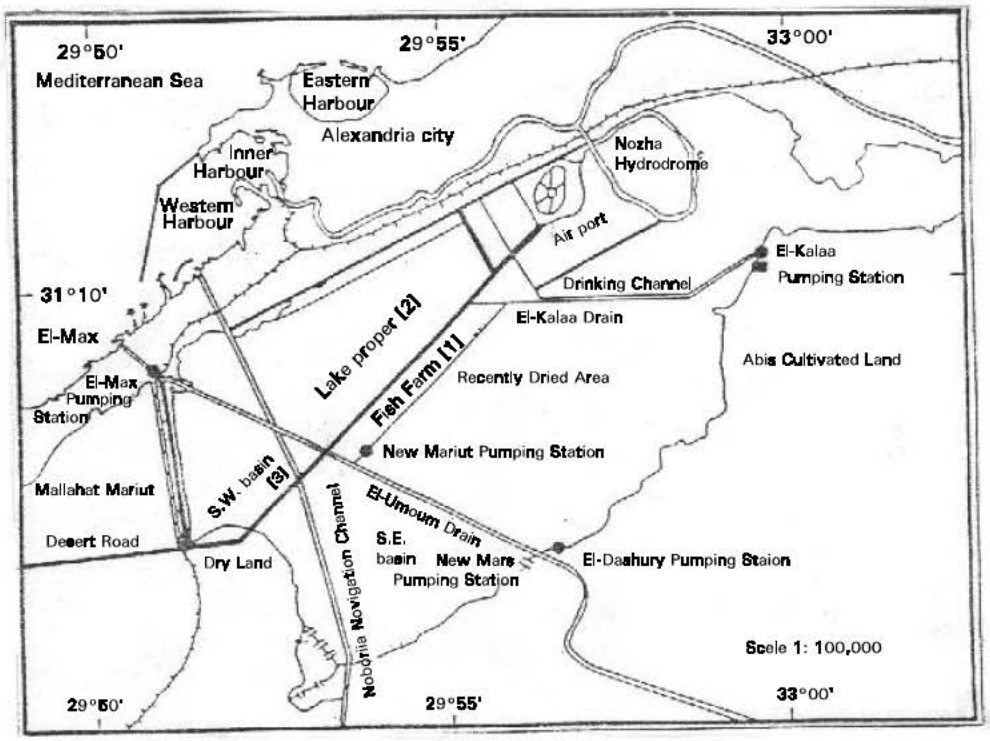
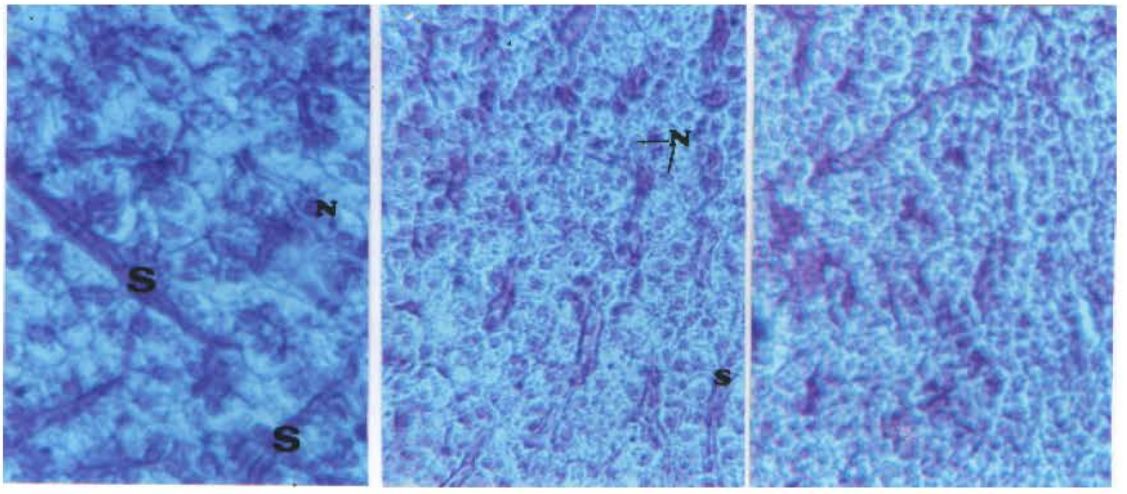
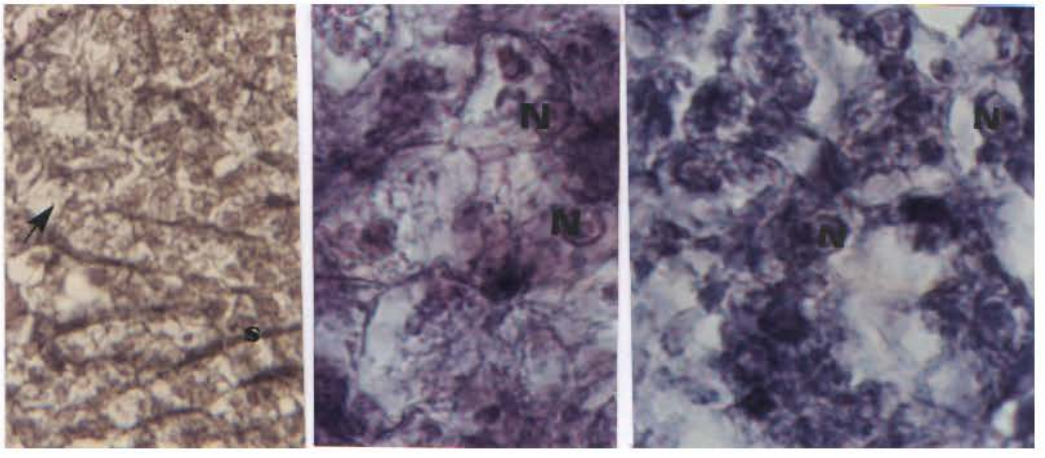


Fig. 1. Study area and sampling locations (1,2 &3)



A B C

Figs. 2 (a, b & c): Liver sections of *T. zilii* , fixed in 10% neutral formalin, stained with mercury bromophenol blue (Hg-BPB), X 1400. a) Illustrate specimen caught from Fish form. The protein inclusions appear at the marginal areas of the hepatocytes, in addition to little patches scattered in the cytoplasm, the hepatic sinusoids (S) are moderalty stained, N: nuclei. b) Moharrem Bey area specimen. The amount of protein granules is found in the intensely stained cytoplasm particularly adjacent to sinusoid (S). c) Merghim basin specimen revealed elevated protein content in the deteriorated tissues.



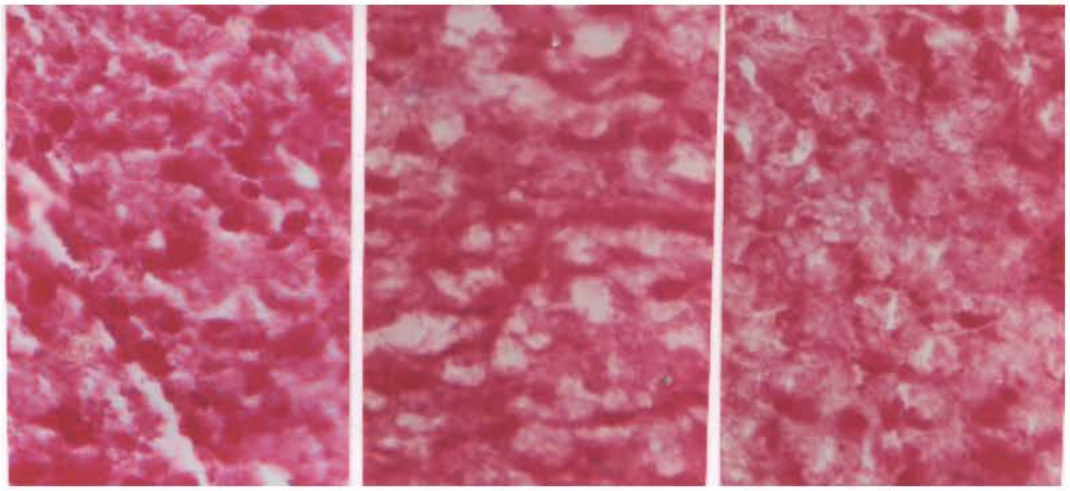
A B C

Figs. (3a, b & c): Liver sections of *T. zilii* fixed in 10% neutral formalin and stained with Sudan black B, X 1400. a) Specimen caught from Fish form revealing fat globules in the cytoplasm of hepatocyte. Arrows pointed at sudanophobic nuclei, s: liver sinusoids. b) Specimen of Moharrem Bey area showing marked increase in the lipoid materials indicated by the high level of Sudan black B reactivity. c) Merghim basin specimens demonstrate distinct lipid inclusion located in hepatocytes mainly in-between degenerative vacuoles as well as peripheral region of hepatocyte.

Discussion

This study using bromophenol blue technique revealed accumulation of liver protein granules in fishes caught from the polluted locations (Merghim basin > Moharrem Bey > Fish farm). An increase in liver protein was recorded by Khan *et al.* (1987b) after application of crude and base oils. On the contrary, Goel *et al.* (1988) illustrated a decrease in hepatic proteins with the administration of n-hexane. In parallel, This study indicated an increase in total serum protein in fish inhabiting areas with high rates of hazardous contamination (heavy metals at Moharrem Bey; heavy metals and petroleum oil at Merghim basin) as compared to reference area; the increase at Moharrem Bey was however, insignificant. The results reported for serum proteins are in agreement with those obtained by McKim *et al.* (1970), who to base oil and crude oil administration. Besides, Goel *et al.* (1988) observed an increase in lipid peroxidation following n-hexane administration.

The abnormal accumulation of fats in experimental animals could be due to induced imbalance between fat production and utilization (Moore *et al.*, 1988). It could be postulated that fatty liver changes are attributed largely to the reduced production of lipoproteins. Thus, the triglycerides, which are normally released as lipotein, accumulate in liver cells (Cappel and Anderson, 1971). The phenomena of fatty liver, or better defined, the storage of large quantities of fats in the fish liver, is quite normal at certain times, including periods of sexual maturation (Kranz and Peters, 1985). A dietary imbalance is another especially common cause of fatty livers in cultured fishes (Hille *et al.*, 1980). However, when the diet is normal, when the fishes are beyond the spawning seasons, and when a concurrent combination with other degeneration phenomena is observed, fatty livers must undoubtedly be considered pathological (Roberts, 1978).



A B C

Figs. 4 (a, b & c): Liver sections of *T. zilii* fixed in 10% neutral formalin, a) Specimen from the Fish farm, Notice: the peripheral position of glycogen granules of variable quantities in neighboring cells. b) Specimen caught from Moharem Bey area with more aggravated glycogen depletion. c) Specimen caught from Merghim with most hepatocytes has lost their glycogen components.

Elevated lipid contents are frequently associated with increased bioconcentration of lipophilic toxicants, which is usually correlated with enhanced toxicity of these compounds (Hickie *et al.*, 1989; Goksφyr *et al.*, 1994).

Furthermore, there have also been reports that high lipid deposits may exert protective effects by removing and inactivating organic chemicals from the metabolism or even actively sequestering them, thus improving toxicant tolerance and resistance (Kruzynski, 1979; Brauffaldi and Cucchi, 1989). Fluctuation of total lipids and cholesterol in serum of fish versus of relative amounts of pollution in each location, tested, were quite striking. A direct correlation does exist between the concentrations of these parameters, on one hand, and the overall sum of contaminants in the tested areas, on the other.

Contradictory results have been reported by El-Elaimy *et al.* (1988) who noticed marked depletion of total lipids and elevation of phospholipids in liver cells, brain and muscle of freshwater fish exposed to dimethoate malathion and hostathion.

Environmental contamination with heavy metals and other sources, determined in Moharrem Bey and Merghim basin could have inactivated enzymes of lipid metabolism as hepatic lipoprotein lipase. This inhibition, in turn, results in the retardation of clearance of lipids from blood. That may interpret the rise in the concentration of these parameters in the polluted locations. Such interpretation takes support from previous literature of Ramadan *et al.* (1988).

In response to poisoning with chromium (Browning, 1969), lead (Tarugi *et al.*, 1982) and copper (Hilmy *et al.*, 1987) hyperlipidemia and hyper cholesterolaemia were reported in different vertebrates).

Significantly, the concentration of blood glucose was mostly high in fish caught foreign Merghim and to a less extent at Moharrem Bey, as compared to those collected from the reference area. This non-specific effect might have arisen as a stores response following the exposure to altered water criteria in the polluted locations (2&3).

The exposure to pollutants might have stimulated the mobilization of liver glycogen, as illustrated cytochemically in the current study using Best's Carmine technique. It seems that the liver reacts with toxic substances by glycogen depletion. The severe diminution of glycogen observed in specimens of Merghim basin location 3) may be due to the combined effects of heavy metals and petroleum oil. Nevertheless, several authors using some other kind of pollutants previously confirmed the drop of glycogen content in the cells of liver. El-Beih *et al.* (1991) reported glycogen depletion in the liver of guinea pig after the administration of an insecticide (Lannate). Similar results were obtained earlier by Banhawy and Ganzuri (1986) working on the effects of other insecticides (lindane, gusathione and tarmaron) on rat liver. Allyl formate and dimethylnitrosamine induced similar results as reported by Dory and Hinton (1988) and Hinton *et al.* (1988), respectively.

The depletion of liver glycogen (glycogenolysis) and the rise in blood glucose levels were reported in fish after exposure to pollutants (Hanke *et al.*, 1983; Gluth and Hanke, 1985). Meanwhile, more exposure time is required for the induction of liver glycogen depletion because glycogenesis may first take place (Hanke *et al.*, 1983; Anderson *et al.*, 1991). In fish this hyperglycaemia stress response is mediated by increase in plasma cortisol and catecholamine (Gross and Wood, 1988).

Data indicate a significant rise in serum creatinine concentration in fish caught foreign heavily and moderately polluted areas (Merghim basin and Moharrem Bey, respectively) in comparison with the reference area (Fish farm).

This effect might be induced by glomerular insufficiency, increased muscle tissue catabolism or the impairment of carbohydrate metabolism (Murray *et al.*, 1990).

It is apparent from this study that disturbance in cytochemical components (glycogen, lipid protein) in hepatocytes and blood serum as a result of pollution reflect disturbance in all metabolism and can be used as marker of pollution.

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