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Induction of Cytochrome P450 1A1 as a Biomarker of Benzo-a-pyrene Pollution in Egyptian Fresh Water Fish

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Abstract: The activity of Ethoxyresorufin-o-dealkylase (EROD) in the liver of *Oreochromis niloticus* and *Clarias gariepinus* was evaluated as a response to experimental and natural contamination of water with Benzo-a-pyrene and/or cadmium. The activity was measured fluorimetrically in the hepatic S9 fraction while the content of the enzyme was measured by ELISA. The response appeared as early as six hours post exposure. This study also reveled that *Oreochromis niloticus* exhibits higher values of EROD activity than that of *Clarias gariepinus*. CYP450 1A1 content showed lower responsiveness when compared to EROD activity measurements. The present study also estimated the inhibitory effect of cadmium on CYP450 1A1 induction. The current results demonstrate that EROD activity reflects contamination of water with benzo-a-pyrene as a polycyclic aromatic hydrocarbon compound. Consequently it is a useful biomarker for monitoring this type of pollution.

Key words: Cytochrome P450 1A1, EROD, Cadmium, ELISA, benzo-a-pyrene

INTRODUCTION

Aquatic contamination represents a constant source of public health concern. In Egypt, River Nile is the main water source and exposed to many kinds of pollutants whether biological or chemical. Water quality of the River Nile has been affected by the discharges of agricultural drains, industrial, municipal wastes and dead animals that are thrown in several areas along the River (Aboul-Ela *et al*., 1990; El-Sherbini, 1996). Polycyclic Aromatic Hydrocarbons (PAHs) are widely distributed in both fresh water and coastal marine ecosystems where they have been found to bioaccumulate in several aquatic species. They also represent one of the most significant classes of organic pollution due to their carcinogenic and mutagenic potentials (Holladay *et al*., 1998; Barra *et al*., 2001). Benzo-a-pyrene is one of the PAHs, which is ubiquitously distributed throughout the environment as a consequence of its formation during the combustion of organic matter. Its release to the environment appeared to be through incomplete combustion of gasoline, garbage, or any animal or plant material. Also, it could find its way to the aquatic environment through runoffs, oil spill, industrial effluent and atmospheric disposition (Kira *et al*., 1999; Nogami *et al*., 2000; Shaw *et al*., 2004). Another group of hazardous contaminants are represented by the inorganic trace metals. Since most of the toxic organic chemicals are synthetic compounds that are foreign to biota (i.e., xenobiotic), it is surprising that there are biochemical mechanisms in fish to metabolize these compounds. These mechanisms represent the biotransformation processes which have been differentiated into two major types. Phase I mechanisms of biotransformation include oxidation, reduction or hydrolysis reactions in the cells. These reactions usually increase the polarity of the substrate, making the compound easier to excrete from fish. PAHs are easily metabolized by phase I enzymes to more hydrophilic products like phenols, dihydrodiols, quinones and epoxides (Varanasi and Stein, 1991; Livingstone, 1998). The most important phase I biotransformation reactions in vertebrates are oxidations carried out in the endoplasmic reticulum of cells by Mixed Function Oxidases (MFOs), or more properly termed, Cytochrome P450-associated mono-oxygenases (Goksøyr and Förlin, 1992). In fish, as in most vertebrates, the highest activity of MFOs occurs in liver tissue (Stegeman and Hahn, 1994). Induction of CYP450 1A1 in fish liver has been recognized as an excellent biological indicator of exposure to PAHs, particularly benzo-a-pyrene (Goksøyr and Förlin, 1992; Mdegela *et al*., 2005). Phase II biotransformation reactions include a range of

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conjugation reactions where toxic substrates are bound to biomolecules, such as glucuronic acid, glutamate, sulfate or glutathione (Nimmo, 1987). This conjugation dramatically improves solubility, which then promotes rapid excretion. The induction response of biotransformation enzymes in fish to certain classes of organic contaminants was the main concern of early studies (Arinc and Sen, 1994; Parente *et al*., 2004; Winter *et al*., 2005). So, the current study aims to assessment the effect of cadmium and/or Benzo-a-Pyrene on EROD activity and CYP450 1A1 protein content in two different species of freshwater fish (*Oreochromis niloticus* and *Clarias gariepinus*).

MATERIALS AND METHODS

Experimental fish: *Oreochromis niloticus* and *Clarias gariepinus* (80-100 g) were obtained alive from El-wafaa fish farm, Manyl Sheha, Giza, Egypt. They were transported to the laboratory in large plastic water containers and maintained in the glass aquaria to acclimatize for one week before starting the experiment. Food was not provided throughout the experimental period and also before its beginning by 48 h.

Experiments: A total number of 69 fish from each acclimatized *Oreochromis niloticus* and *Clarias gariepinus* were divided into three groups. The first one contained 24 fish and exposed to 1/10 LC_{50} of cadmium chloride, LC_{50} of $CdCl_2$ was 4.072 mg L^{-1} according to Ahmed *et al*. (1998) for 15 days. The second group (24 fish) represents the control group. After 15 days, three fish from cadmium treated and control groups were sampled and their livers were immediately frozen in liquid Nitrogen to study the effect of cadmium only. Then 1 mg L^{-1} Benzo-a-Pyrene (purchased from SIGMA) was dissolved in 0.5 mL Dimethyl Sulfoxide (DMSO) and added to the previous cadmium-treated group. At the same time, the third group (21 fish) received B-a-P only. The control group received DMSO only. Three fish were sampled randomly from each group (control, B-a-P treated and cadmium+B-a-P) at 6, 8, 12, 24, 48, 72 and 96 h after exposure to Benzo-a-Pyrene and livers were immediately frozen in liquid nitrogen until further studies.

Determination of EROD activity: 0.5 g of each liver was homogenized separately in cold O.I M phosphate buffer (pH 7.4) with 10% glycerol. The homogenates were centrifuged at 9000 x g for 30 min at 4°C (Hodson *et al*., 1991) and their protein content were measured according to Lowry *et al*. (1951). Then, EROD activity was determined according to Hodson *et al*. (1991). The fluorescence was measured by spectroflorometer (JASCO EP777, Japan) at excitation/emission wavelengths of 530/585 nm.

Immunodetection of Cytochrome P450 lA1 content in fish liver samples

Preparation of specific antibodies to CYP450 1A1: Two rabbits were immunized subcutaneously with ~40 μg of the enzyme protein (Cytochrome P450 1A1 isozyme, purchased from SIGMA) in Freund's complete adjuvant. A booster dose of ~40 μg protein in Freund's incomplete adjuvant was given 15 days later. Two additional doses of the same protein content and without adjuvant were also administered on day 21 and 28. Serum samples were collected 4 days after last immunization (Fagbemi *et al*. 1995).

ELISA: ELISA was adopted to determine the level of CYP450 1A1 in S9 fraction samples using the prepared antibodies according to Goksøyr (1991).

Random water and fish samples: Random samples were collected from five different locations of the River Nile: Tourrah, south of Cairo; El- Bahren Inland, Pharaonic village at Giza; El-Maryouteyah canal, Nahia, Giza; El-Manyal and Banha. These locations are exposed to different industrial, agricultural and biological pollution.

Determination of cadmium level in water: About 50 mL of the water specimen was acidified with concentrated nitric acid (5 mL L^{-1}) to pH less than 2.0 and kept at -18°C for 1-2 days until cadmium analysis using graphite atomic absorption method (APHA, 1992).

Determination of B-a-P level in water: About 300 mL of each water specimen was lyophilized and extracted by acetonitrile. Benzo-a-Pyrene was determined in the extract using HPLC according to Nogami *et al*. (2000).

Determination of cadmium level in fish samples: About 25 g of pooled fish muscle samples (fresh weight) were dried, asched in a muffle furnace and then dissolved in 1N HCl to determine cadmium residues by stabilized temperature graphite furnace atomic absorption spectrometry (AOAC, 1980).

Determination of B-a-P level in fish samples: About 10 g of muscle fish tissues were homogenized with an extraction solution hexane-acetone (1:1, v/v), centrifuged and the supernatant was then carefully evaporated by rotatory vacuum evaporator at 40°C just to dryness. Residue was dissolved in acetonitrile (HPLC gradiant) and Benzo-a-Pyrene residues were determined by HPLC (Janska *et al*., 2004).

RESULTS

A time-dependent response of hepatic EROD activities after exposure to 1 mg L^{-1} B-a-P was proved in the current study. EROD began to increase after 6 h of B-a-P exposure (32.6 pmol/min/mg protein in case of *Oreochromis niloticus* and 12.3 pmol/min/mg protein in case of *Clarias gariepinus* and reached its maximum value at 48 h (1055.6 and 73.9 pmol/min/mg protein in case of *Oreochromis niloticus* and *Clarias gariepinus* respectively) as depicted in Fig. 1. Then, these values began to decrease till reach 62.2 and 5.2 pmol/min/mg protein in case of *Oreochromis niloticus* and *Clarias gariepinus*, respectively at 96 h, but still higher than that of control values (Fig. 1). The present study also reveled that *Oreochromis niloticus* exhibits higher values of EROD activity than that of *Clarias gariepinus* (Fig. 3).

CYP450 1A1 content, measured by ELISA, showed lower responsiveness when compared to EROD activity measurements after B-a-P exposure in both studied fish species (Fig. 2). There was 215.43 fold increases in EROD activity compared to 1.37 fold increases in CYP450 1A1 content in case of *Oreochromis niloticus* treated with B-a-P for 48 h. While in case of *Clarias gariepinus* there were 13.26 fold increases in EROD activity and 1.47 fold increases in CYP450 1A1 content at 48 h.

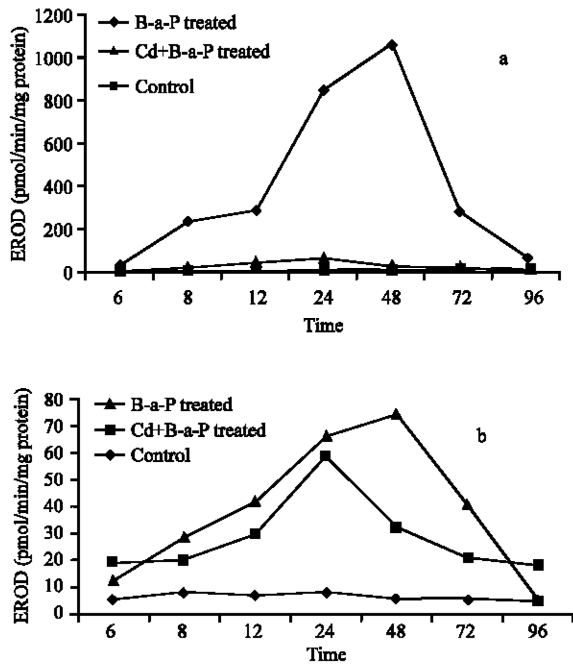

Fig. 1: Effect of Benzo-a-Pyrene and/or cadmium on EROD activity of a) *Oreochromis niloticus* and b) *Clarias gariepinus* at different time intervals

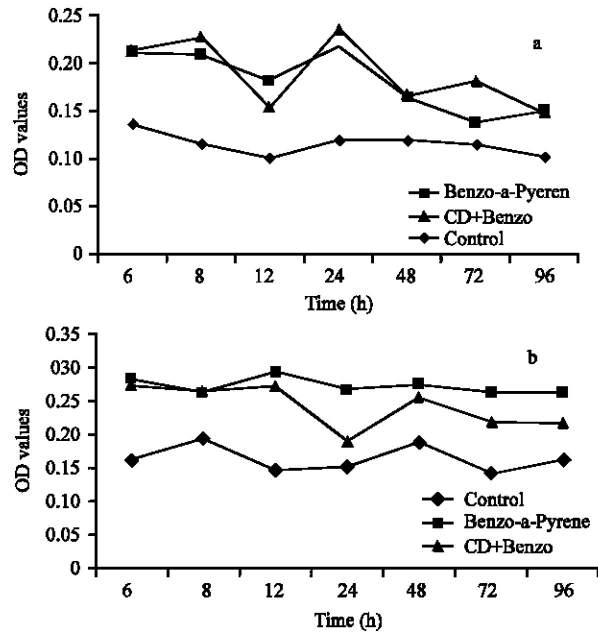

Fig. 2: Effect of Benzo-a-Pyrene and/or cadmium on CYP450 1A1 content of a) *Oreochromis niloticus* and b) *Clarias gariepinus* at different time intervals

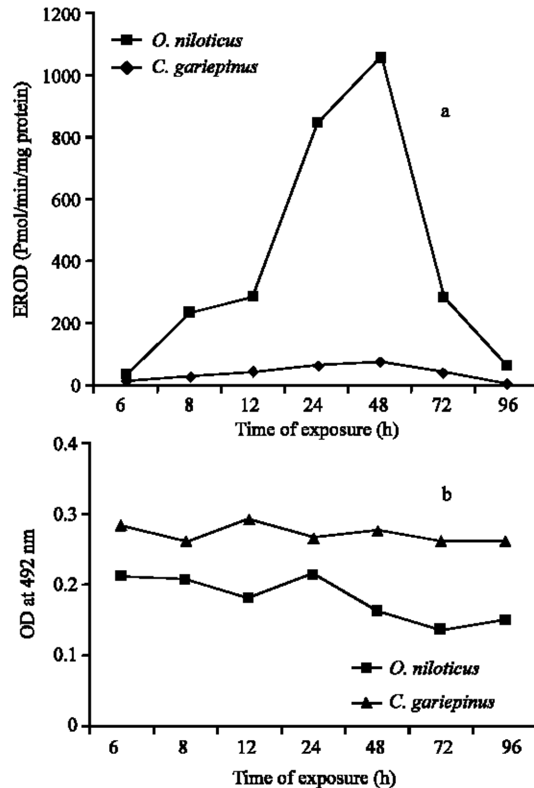

Fig. 3: Comparative effect of Benzo-a-Pyrene on a) EROD activity and b) CYP450 1A1 content of *Oreochromis niloticus* and *Clarias gariepinus*

Experimental exposure to Cd Cl_2 for 15 days decreased EROD activities in both studied fish species (Fig. 4). While pre-exposure to Cd Cl_2 for 15 days lowered the effect of Benzo-a-Pyrene on EROD activities. Concerning Cd Cl_2 effect on CYP450 1A1 content in *Oreochromis niloticus* and *Clarias gariepinus*, the present study revealed a slight increase in both species when exposed to cadmium chloride only for 15 days (Fig. 5). Pre-exposure to cadmium chloride for 15 days lowered the effect of B-a-P on CYP content of *Clarias gariepinus* when compared with the effect of B-a-P only. However cadmium pre-exposure effect on CYP450 1A1 content of *Oreochromis niloticus* showed no proportional correlation with the effect of B-a-P only (Fig. 5).

Regarding field studies on random fish and water samples, the present study recorded the highest level of B-a-P in El-Mariotya canal (1.8±1.2 ng L^{-1}), followed by Toura, Banha, Pharaonic village and El-Manyl which recorded 1.062±0.42, 0.502±0.26, 0.215±0.09 and 0.196±0.16 ng L^{-1}, respectively (Table 1). The highest level of cadmium was also recorded in El-Mariotya canal (2.112±0.71 μg L^{-1}). It was followed by 0.927±0.22, 0.354±0.05, 0.156±0.04 and 0.004±0.01 μg L^{-1} in Banha, El-Manyl, Toura and Pharaonic village, respectively (Table 1).

Concerning Cytochrome P450 1A1 induction in random fish samples collected from the different studied locations, the highest level of hepatic EROD activity during this study was recorded in fish samples from Toura and Pharaonic village (246.9±56.9 and 229.2±165.3 Pmol/min/mg protein) as shown in Table 1. While fish samples from El-Mariotya canal and Banha have low EROD activity levels; 148.1±91.4 and 14.1±6.7 Pmol/min/mg protein, respectively (Table 1). Concerning CYP450 1A1 content of random fish samples, the current study revealed similar results in all locations except that in El-Mariotya which showed lower value than other locations OD = 0.268±0.04 (Table 1).

B-a-P was not detected in fish muscles of neither Pharaonic village nor El-Manyl (Table 1). While there were small levels (1.660±1.54, 0.127±0.07 and

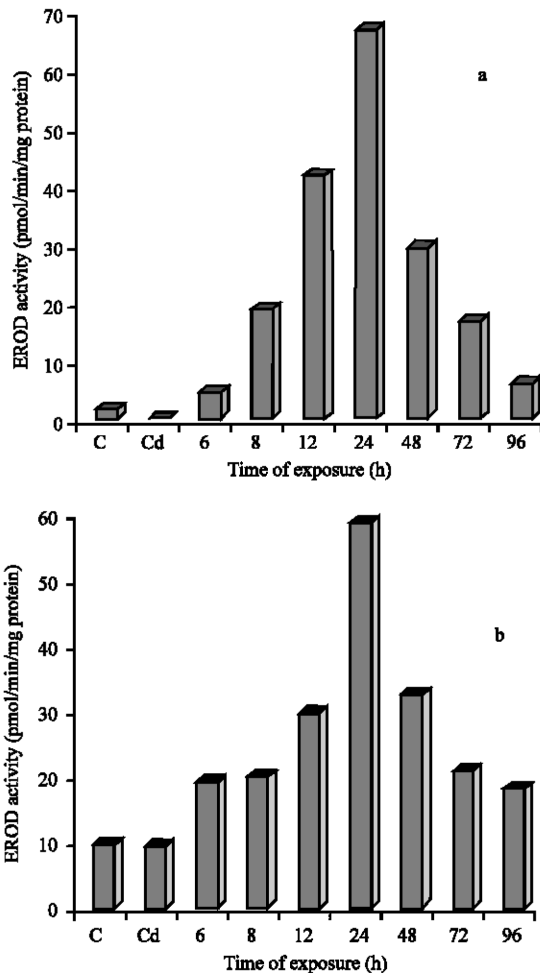

Fig. 4: Effect of cadmium and cadmium + Benzo-a-Pyrene on EROD activity of a) *Oreochromis niloticus* and b) *Clarias gariepinus* at different intervals

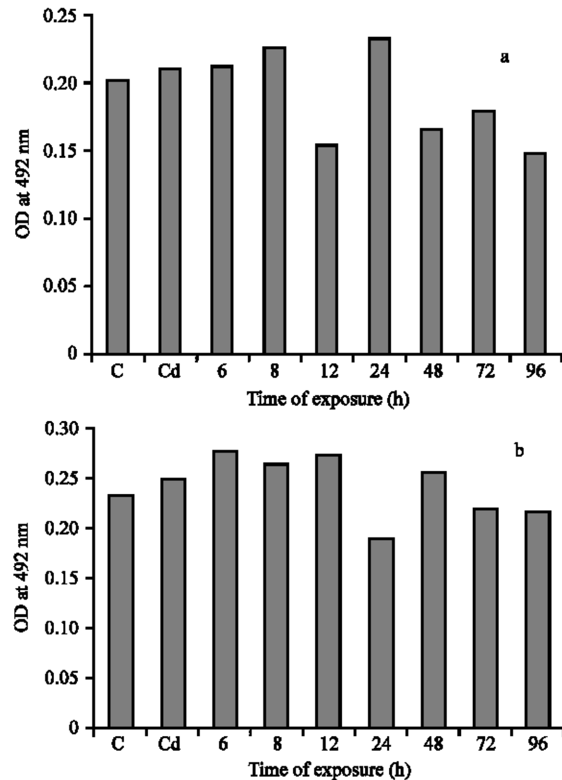

Fig. 5: Effect of cadmium and cadmium + Benzo-a-Pyrene on CYP450 1A1 content of a) *Oreochromis niloticus* and b) *Clarias gariepinus* at different intervals

Table 1: B-a-P and Cd levels in water and fish muscle randomly collected samples in relation to CYP450 1A1 induction in fish

Location	B-a-P in water (ng L^{-1})	B-a-P in muscles (ng g^{-1})	Cd in water (μg L^{-1})	Cd in muscles (μg g^{-1})	EROD activity (Pmol/min/mg protein)	CYP450 1A1 content (OD values)
Pharaonic village	0.215±0.09	ND	0.004±0.01	0.019±0.01	229.2±165.3	0.411±0.06
El-Manyl	0.196±0.16	ND	0.354±0.05	0.037±0.01	32.7±11.9	0.411±0.06
Toura	1.062±0.42	0.127±0.07	0.156±0.04	0.027±0.01	246.9±56.9	0.415±0.05
El-Mariotya	1.804±1.16	1.660±1.54	2.112±0.71	0.038±0.01	148.1±91.4	0.268±0.04
Banha	0.502±0.26	0.004±0.00	0.927±0.22	0.063±0.02	14.1±6.7	0.438±0.09

0.004±0.00 ng gm^{-1} wet weight) in fish from El-Mariotya, Toura and Banha, respectively (Table 1). Cadmium levels in muscles of the studied fish samples were also recorded. They were 0.063±0.02 (μg gm^{-1} wet weight) in Banha, followed by 0.038±0.01, 0.037±0.01, 0.027±0.01 and 0.019±0.01 (μg gm^{-1} wet weight) in El-Mariotya, El-Manyl, Toura and Pharaonic village, respectively (Table 1).

DISCUSSION

The effect of one of the most serious PAHs, Benzo-a-Pyrene, on fish CYP1A1 was previously assessed (Zapata-Perez *et al*., 2002; Gorbi and Regoli, 2004; Mdegela *et al*., 2005). In this study, the effect of Benzo-a-Pyrene (B-a-P) on CYP450 1A1 was estimated in both *Oreochromis niloticus* and *Clarias gariepinus*. Concerning hepatic EROD activity, it was found that there was a time-dependent effect on hepatic EROD activities after exposure to 1 mg L^{-1} B-a-P. EROD began to increase after 6 h of B-a-P exposure and reached its maximum value at 48 h. After that, these values began to decrease, but still higher than that of control values. More or less similar response on EROD activities was previously recorded in different fish species as a result of exposure to B-a-P. As in Arctic charr; *Salvelinus alpinus* (Wolkers *et al*., 1996), in gizzard shad; *Dorosoma cepedianum* (Levine and Oris, 1997) and in European eel; *Anguilla anguilla* (Gorbi and Regoli, 2004). Induction of CYP450 1A1 may be explained as a result of binding of foreign compound (xenobiotic) to a cellular receptor (Perdew and Poland, 1988), often called the Aryl hydrocarbon or Ah receptor. This binding triggers the expression of the gene coding for CYP450 1A1 leading to increase RNA transcription (Okey *et al*., 1994) and hence increase synthesis of CYP450 1A1. EROD activity is considered to be specifically catalyzed by the cytochrome P450 1A1 gene product which is inducible by aromatic and chlorinated hydrocarbons (Stegeman, 1989; Goksøyr *et al*., 1991). Hepatocytes were also retaining their capacity to induce CYP450 1A1 for several days by maintaining the Ah receptor in cells (Lorenzen and Okey, 1990). This information explains the response of EROD activity to B-a-P that introduced in the current research.

During the present study, there was a lower response of CYP450 1A1 content, measured by ELISA, compared to EROD activity measurements after B-a-P exposure in both studied fish species. These results were also similar to that recorded by Lemaire-Gony and Lemaire (1992) and Wolkers *et al*. (1996) who estimated that exposure to B-a-P had no detectable effect on microsomal CYP450 content. It was also similar to that of Zapata-Perez *et al*. (2002) who estimated 1.9 fold increases in CYP4501A protein in Tilapias when exposed to pyrene compared to 18 fold increase in EROD activity under the same conditions. This difference between the response of EROD activity and CYP450 1A1 content to pollutants may be explained by the suggestion that the increase in catalytic activity does not necessarily indicate a new synthesis of enzyme protein (Jimenez and Stegeman, 1990).

It was noticed from the current study that *Oreochromis niloticus* exhibits higher values of EROD activity than that of *Clarias gariepinus*. In Egypt, as in most countries, Tilapias are among the species most frequently found in fish farming activities, in the River Nile and its tributaries. Besides its wide geographic distribution and abundance, an additional advantage of using *O. niloticus* for monitoring purposes is its well known resistance to highly polluted environments (Parente *et al*., 2004) and this may explain the higher values of EROD activities in case of *Oreochromis niloticus* than that recorded in *Clarias gariepinus*.

On the other hand, CYP450 1A1 content of *Clarias gariepinus* was higher than that of *Oreochromis niloticus*. This result ensure the opinion of some authors that EROD induction does not necessarily accompanied by increase in CYP450 1A1 content (Jimenez and Stegeman, 1990; Lemaire-Gony and Lemaire, 1992; Wolkers *et al*., 1996). Also *Clarias gariepinus* considered as a muddy bottom and carnivorous fish which make it in a continuous exposure to different pollutants which concentrated in the sediment. Biomagnification of pollutants through eating of animals which already accumulate chemicals make *Clarias gariepinus* accumulate pollutants in greater amount than in case of other herbivorous fish; *Oreochromis niloticus*. These differences in backgrounds of the two studied species may have some impact on the responses and may lead to a certain level of adaptation to the pollutants as recommended by Otto and Moon (1996). This may explain the increased basal level of CYP450 1A1 protein in *Clarias gariepinus* rather than in *Oreochromis niloticus*.

Heavy metals and hydrocarbons are widely dispersed pollutants in the aquatic environment (McElroy *et al*., 1989; Zelikoff, 1993) and are generally present together in polluted areas. This study revealed that pre-exposure to 1/10 LC_{50} of $Cd\,Cl_2$ for 15 days, decreased the effect of B-a-P on EROD activities in both studied species. Several authors have reported similar inhibition of fish phase I activities after exposure to cadmium (Gagne and Blaise, 1996; Al-Arabi and Goksøyr, 2002). Cadmium was detected also as the most effective metal on EROD activity that cause 50% inhibition of EROD activity at a significantly lower concentration (2.2×10^{-5} M) (Bruschweiler *et al*., 1996). Inhibition of CYP1A1 induction may be interpreted as a consequence of the global effects of cadmium on the cells. Cadmium exposure alters the membrane structure of fish hepatocytes after long-term exposure (Lemaire-Gony and Lemaire, 1992). Heavy metal can bind with membrane proteins and phospholipids and modify the charge structure on the surface and the intrinsic conformation of the lipid molecules (Cvec, 1990; Tocanne and Tessie, 1990). Among the proteins which are expected to be vulnerable to free metals to interact with them, are hemoproteins. Due to the high affinity of metals to SH-residues, they could also react with various cellular ligands, making complexes with SH-containing molecules such as the thiol group of the CYP cystein which is linked to the heme iron (Omura *et al*., 1993; Risso-de Faverney *et al*., 2000). The accelerated heme turnover caused by metals may substantially depress the oxidative function of the cell, including that of the protective microsomal cytochrome P450 monooxygenase system (Sauer, 1987; Manelis *et al*., 1993). However these results are in contrary to those of Lemaire-Gony *et al.* (1995) who detected an increase in EROD activity of sea bass (*Dicentrarchus labrax*) when exposed to 40 $\mu g\ L^{-1}$ cadmium for 15 days before injection with 20 mg Kg^{-1} B-a-P.

Concerning B-a-P and cadmium levels in random water and fish samples, the highest level of B-a-P was recorded in El-Mariotya canal. This location represents a small branch of River Nile which receives agricultural and biological pollution coming from Zeinene drainage. Bad habits of some people in this location as, burning of garbage and dead animals and plants, also represent a main reason of high level of B-a-P (IARC, 1983; ATSDR, 1995; PHG, 1997). Toura location represents a wide part of River Nile which passes through Toura city and receives industrial pollutants. The high B-a-P level in this location may be attributed to high levels of boat traffic. This location was similar in its conditions to that mentioned by Shaw *et al.* (2004). B-a-P level in Banha may be attributed to the presence of coal factory near to that location which burning wood to get coal. Pharaonic village receives pollution from fuel of fishermen boats aggregated in this location; While El-Manyl receives somewhat lower pollutants. B-a-P is one of the PAHs which are hydrophobic compounds with low water solubility and therefore, their concentrations in water are very low (Nestervova *et al*., 1982; El Nemr and Abd-Allah, 2003) and they tend to adsorb on the suspended material and sediments.

The highest level of hepatic EROD activity during the current study was recorded in fish samples from Toura and Pharaonic village (246.9 and 229.2 Pmol/min/mg protein). It was clearly noticed that these locations recorded considerable levels of B-a-P (1.062 and 0.215 ng L^{-1}, respectively) and also recorded low cadmium levels (0.156 and 0.004 $\mu g\ L^{-1}$, respectively). Although El-Mariotya canal and Banha have high levels of B-a-P (1.8 and 0.502 ng L^{-1}) they have low EROD activity levels (148.1 and 14.1 Pmol/min/mg protein, respectively). This lack of CYP450 1A1 induction may be due to inhibition caused by high level of cadmium in these sites (2.112 and 0.927 $\mu g\ L^{-1}$, respectively). This was in accordance with results estimated by Tuvikene *et al.* (1999).

Concerning B-a-P prevalence in muscles of random fish samples, it was found that B-a-P was not detected in neither Pharaonic village or in El-Manyl. It has small level (1.660, 0.127 and 0.004 ng g^{-1} wet weight) in El-Mariotya, Toura and Banha respectively. These results come in accordance with the opinion that the elimination of PAHs is generally very efficient in fish; no bioaccumulation of these compounds has generally been demonstrated. This is due to the chemical oxidation or biological transformations by phase I enzymes to more hydrophilic products like phenols, dihydrodiols, quinones and epoxides (Varanasi and Stein, 1991; Livingstone, 1998). Furthermore the rate of pollutant distribution in different organs depended on the regional blood flow through each organ so, organs which have a high blood flow (liver and kidney) tend to accumulate xenobiotics most readily than that of a low blood flow (muscles) (Pritchard, 1993).

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