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Effect of Polycyclic Aromatic Hydrocarbons (PAHs) on Modulate Genes Encoding Stress Related Proteins and Antioxidant Enzymes in Different Marine Fish Species of Red Sea Water

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) in the Red sea water were determined at Suez and Ismailia governorates, Egypt. The sites were selected to represent areas with different activities of Suez Gulf water. The concentrations of fifteen PAHs having two to six rings were determined by using HPLC with florescence detection. The total average concentrations of the fifteen PAHs over the Suez governorate sites (S1 and S2) were 1.45 and 0.34 µg/l, respectively. However those for Ismailia governorate sites (I1 and I2) were 1.06 and 0.24 µg/l, respectively. PAHs having four to six rings were the predominant compounds in particulate matters. The major sources of PAHs and their impacts on the health of certain types of fish such as Mullet and Sea-bass species were studied through the effect on the expression of stress protein related genes. In addition, expression of stress protein related genes (Cytochrome P450, CYP1A and metallothionein, MT-1A) and antioxidant enzymes (Glutathione-S-Transferase and GST alpha) in liver tissues of Mullet and Sea-bass collected from studied locations in Ismailia governorate and Suez gulf were assessed. The results revealed alterations in the hepatic mRNA levels of CYP1A, MT-A and GST alpha genes in Mullet and Sea-bass collected from S1 location at Suez gulf compared with I1 location at Ismailia governorate and with the S2 and I2 locations as reference site. The current findings suggest that the genes encoding stress related proteins and antioxidant enzymes studied in this paper represent valid biomarkers to detect variation of fish stress conditions attributed to PAHs.

Key words: Polycyclic Aromatic Hydrocarbons · Cytochrome P450 · Antioxidant Enzyme · Marine Fish Species · Red Sea

INTRODUCTION

Egypt is growing not only in population and number of vehicles but also in industrial activities. Urbanization and industrialization have increased very rapidly in Egypt, particularly in the second half of the last century, causing an increase in the pollution of its air-water environment [1]. Polycyclic aromatic hydrocarbons (PAHs) are among the most carcinogenic, mutagenic and toxic contaminates found in aquatic systems [2]. PAHs are a wide spread class of environmental chemical pollutants. There are several possible sources for PAHs in the environment. Two classes of PAH inputs must be considered as natural processes and anthropogenic activities, but the later is generally considered to be the major source of PAH input into the environment [3, 4].

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Although some PAHs are naturally occurring, the majorities are anthropogenic and enter the environment through release of petroleum products (petrogenic sources) or by combustion of organic matter (pyrogenic sources). Recent studies have shown that pyrogenic sources predominate in urban settings and that the profile of PAHs in urban storm water resembles that of atmospheric deposition [5]. However the NPAHs in the two phases have not been compared.

The discharge of PAHs from urban watersheds is exacerbated in arid regions. Arid urban watersheds have a tremendous number of sources. Moreover, the long antecedent periods without rain in arid regions potentially enhance the dry deposition of PAHs to urban landscapes from these atmospheric sources. When rainfall does occur, the precipitation is often short but intense. Runoff from these largely impervious urban surfaces efficiently mobilizes deposited material, including PAHs, in the resulting surface runoff [6].

Due to the change from animal protein to fish protein with reduced cholesterol levels the global consumption of aquatic species and derived fish products has generally increased during the recent decades [7]. It has been predicted that fish consumption in developing countries will increase by 57%, from 62.7 million tons in 1997 to 98.6 million in 2020. However, growing demand for aquatic products in both developing and developed countries has necessitated the need to maintain the present per capita supply of aquatic products in the future. Besides, practices such as environmental perturbations, overexploitation, dumping of agrochemicals and industrial pollutants in the aquatic systems not only reduce abundance and fish size but also pose health hazards to the consumers [8].

Moreover, massive amounts of domestic wastewater and industrial effluents are transported by rivers and discharged into the sea, contaminating coastal waters. Such anthropogenic pollutants are the main sources of heavy metal contaminants in the ocean [9]. Fishes, being one of the main aquatic organisms in the food chain, may often accumulate large amounts of contaminants. Among the animal species, fishes are the inhabitants that cannot escape from the detrimental effects of these pollutants [10]. Aquaculture industries are also exposed to many chemical and biological pollutants [11].

Polycyclic aromatic hydrocarbons (PAHs) listed by the US Environmental Protection Agency as priority pollutants, are widespread organic pollutants in the environment which are well known for their mutagenic and carcinogenic effects and bioaccumulation in animal and human tissues. The sources of PAHs are both natural and anthropogenic, mainly from incomplete combustion of organic materials, fossil fuel and petroleum [12]. PAHs can also form in meat cooked or fish smoked at high temperatures. Falcó *et al*. [13] reported that the main reason for concern following human exposure to these compounds is that several PAHs are carcinogenic and so on. Various recent epidemiological studies have revealed that dietary exposure to PAHs is associated with an increased risk for some human cancers [14]. According to Wang *et al.* [15], the fish feeds might be the main source of the enriched PAHs in the aquaculture sediments.

The present study was carried out to identify the PAH-contaminated locations in the Red Sea including Gulf of Suez and Ismailia Governorate and study its effect on the fish health. Our previous study indicated that some locations in the gulf are heavily contaminated with PAHs which affect some of the resident fish such as the bass (*Epinephelus guaza*) [16]. Therefore, the main goal of the current work is to identify if PAHs contamination of other locations of Gulf of Suez and Ismailia affect fish health such as Mullet and Sea-bass species. This was addressed by determination of the baseline value of number of measurements considered to be indicative of oxidative stress. Since exposure to PAHs is associated with alteration of the stress related proteins such as heat shock proteins (HSPs) [17], we also added them to our baseline measurements. Base on this act, molecular approach by measuring the gene expression of stress-related protein (metallothionein, MT-1A and Cytochrome P450, CYP1A) as well as levels of mRNAs of antioxidant enzyme, Glutathione-S-Transferase, (GST alpha) in liver of Mullet and Sea-bass in response to polycyclic aromatic hydrocarbons were studied.

MATERIALS AND METHODS

Chemicals: EPA610 PAH Mixture, containing naphthalene (Nap), acenaphthalene (Ace), fluorine (Fle), anthracene (Ant), phenanthrene (Phe), fluoranthene (Frt), pyrene (Pyr), benz [*a*] anthacene (BaA), chrysene (Chr), benzo [*b*] fluoranthene (BbF), benzo [*k*] fluoranthene (BkF), benzo [*a*] pyrene (BaP), dibenz [*a,h*] anthracene (DBA), benzo [*ghi*] perylene (BghiPe) and indo [*1,2,3-cd*] pyrene (IDP) were purchased as a PAH standard solution from Supleco (Bellefonte, PA, U.S.A). Five deuterated PAHs (Nap-d_8, Ace-d_{10}, Phe-d_{10}, Pyr-d_{10} and BaP-d_{12}) were purchased from Wako Pure Chemical (Osaka, Japan) as internal standards and they were dissolved in acetonitrile (Kanto Chemical, Tokyo, Japan).

Fig. 1: Water and Fish sampling sites

Table 1: Longitude and latitude of the sampling sites at Suez and Ismailia governorate

Sampling sites	Longitude	Latitude
Suez governorate (S1 & S2)	29.9833° N	32.5500° E
Ismailia governorate (I1 & I2)	30.6000° N	32.2833° E

Sampling Sites Description: Suez and Ismailia are a governorates located on the Red sea and considered as a part from Suez Canal area that includes Suez, Ismailia and Port-Said governorates. This area has heavily polluted water especially near the ports due to presence of a lot of oil ships passing every day, ship maintenance centers and many industrial and oil facilities around the Suez canal.

Six sampling stations were selected three along each governorate coast. The Suez sites are S1, just 1km from Suez port and S2 at a distance of 10km from the port. These sites are affected mainly by the passing and oil discharging of the oil ships along the canal and the industrial waste discharging from the ship maintenance centers and oil companies around the canal. Whereas, the Ismailia sampling sites are I1 and I2. I1 is very close to the Ismailia port and I2 is about 10km from the port. These sites are affected by the motor ships and the urban activities as well (Fig. 1, Table 1).

Fish samples, mullet (*Mugil cephalus*) and seabass (*Dicentrarchus labrax*), were collected in the period between May to July of 2013, from I1 and I2 at Ismailia governorate and SI and S2 at Suez gulf, Egypt. The samples (n=20 per location) were collected, killed and kept immediately on icebox. The fishes were transported in icebox to the laboratory and the analyses were started after approximately 2 hours of the collection. Afterwards, liver tissues from each fish within each location were kept in liquid nitrogen until molecular biological analysis.

Water Sampling and Extraction: Surface water samples (20 cm depth) were collected using narrow neck glass bottles each of two liters volume. Just after sampling, 100 mL (5%) of methanol were added to each sample. The solutions were filtered through a glass fiber filter paper (GC-50, 0.45 µm poor size) followed by using a 3M embore disc filtration (C18, 47 mm poor size). C18 discs were extracted using dichloromethane followed by super sonication twice, filtration through a glass filter paper, addition of 100 µm DMSO, evaporation followed by dissolving in 900 µl ethanol and then evaporation. Finally the sample solution was filtered through a membrane filter (HLC-DISK13). Other conditions were the same as in our previous paper [3, 5, 18].

PAHs Analysis: Fifteen PAHs were analyzed by using HPLC with fluorescence detection. The mobile phase was a mixture of acetonitrile and water with a gradient concentration mode of acetonitrile. The flow rate was 1 mL/min. The time program of the fluorescence detector was set to detect at optimum excitation and emission wavelength for each PAH. NPAHs were analyzed by using HPLC with chemiluminescence detection with several modifications according to our laboratory detection method [5, 19].

The validity of this method were already confirmed through our previous published reports showing the recoveries varied from 87 to 104%, limits of detection (S/N = 3) varied from 0.25 to 1.5x 10^{-15} mol and limits of quantification (S/N = 10) varied from 10^{-15} to 10^{-12} mol (over two orders) and showed a good linearity ($r^2 \geq 0.899$) [4, 5].

Gene Expression Analysis

Isolation of Total RNA: TRIzol® Reagent (Invitrogen, Germany) was used to extract total RNA from liver tissues of mullet (*Mugil cephalus*) and seabass (*Dicentrarchus labrax*) according to the manufacturer's instructions. Isolated total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel

Table 2: Primer sequences used for qRT-PCR

Gene	Primer sequence (5′-3′)[a]	References
CYP1AMullet	ACATCACAGACTCCCTCA ---------- CTCCTGTATCTCTGGGTAA	An *et al.* [22]
CYP1A Sea bass	TTTGGTGGCCTTGACAACAG ---------- CTCCAGCACATTCCCAATGA	Acc number: U78316
Metallothionein (MT-1A) Mullet	CAAGTGCTCCAACTGTGCAT TACACCAGGCCTCACTGACA	Acc number: BT059876
Metallothionein (MT-1A) Sea bass	GCGAGTGCTCTAAGACTGGA ---------- ACTGGCAGCAGCTAGTGTCG	Man and Woo [23]
GST alpha Mullet	GCT CAC AAG AAG TTT CCG ACA GTC CCC AGG CAC CTT TTA TAC TA	Acc number: EZ790503.1
GST alpha Sea bass	GGGGAGGGAGAATGGAGTC CTTCTCCTCCAACATCAG	Kim *et al.* [24]
β-actin	GTGATGAAGCCCAGAGCAAGA TGGTCACAATACCGTGCTCAAT	An *et al*. [22]

[a]F: forward primer; R: reverse primer.

electrophoresis (data not shown). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

Reverse Transcription (RT) Reaction: Complete Poly (A)$^+$ RNA isolated from liver tissues of mullet and sea bass was reverse transcribed into cDNA in a according to Ali *et al.*, [20].

Real Time- PCR (qPCR): QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the liver cDNA copy number from *mullet* and sea-bass. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix ExTaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water and 5 µL of cDNA template [21]. Each experiment included a distilled water control. The sequences of specific primer of the genes used are listed in Table 2.

At the end of each qPCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers.

Calculation of Gene Expression: First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet: $Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method if Ef for the target (CYP1A, metallothionein and GST alpha) and the reference primers (β-Actin) as follows: $\Delta C_{T(test)} = C_{T(target, test)} - C_{T(reference, test)}$, $\Delta CT(calibrator) = C_{T(target, calibrator)} - C_{T(reference, calibrator)}$, $\Delta\Delta CT = \Delta C_{T(Test)} - \Delta C_{T(calibrator)}$. The relative expression was calculated by $2^{-\Delta\Delta CT}$.

Statistical Analysis: All results were expressed as mean ± SE of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 11 followed by least significant difference (LSD) to compare significance between groups. Difference was considered significant when $P > 0.05$.

RESULTS

In this study, the concentration levels of PAH at Suez Gulf sites within Suez and Ismailia governorates were determined. The total average concentrations of the fifteen PAHs in S1 and S2 1.4×10^3 and 0.24×10^3 ng/l, respectively, while those for I1 and were 1.05×10^3 and 0.34×10^3 ng/l, respectively (Fig. 3A). The individual PAH concentrations showed higher values at Suez Gulf sites in Suez governorate (S1 and S2) than those of Ismailia governorate sites (I1 and I2) with the decreasing order of S1>S2; I1> I3 and S1> I1>> S2 > I2 (Table 3).

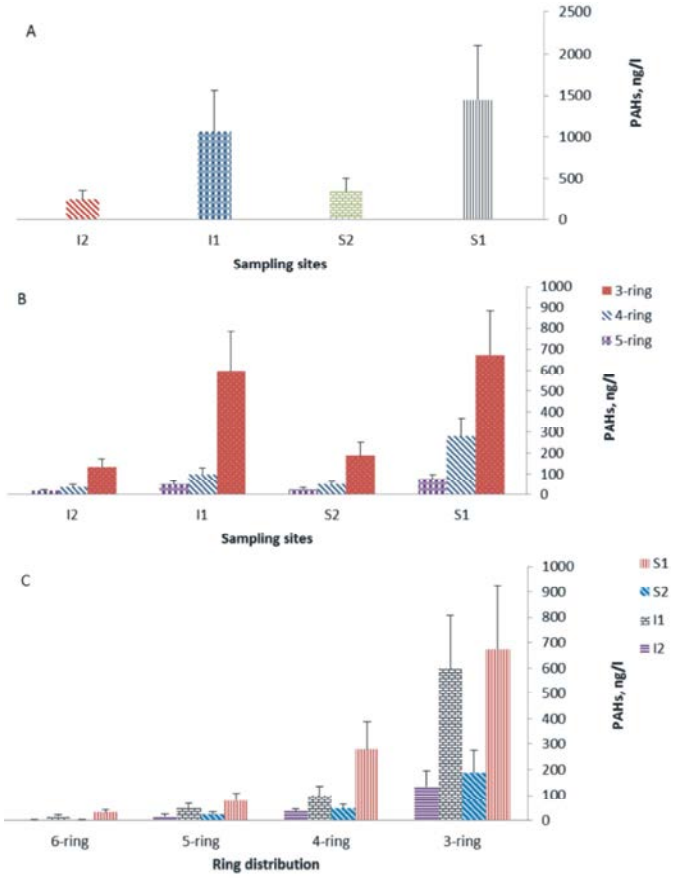

Fig. 2: Comparison between total averages PAH concentration levels at Suez and Ismailia sampling sites (A); PAH ring distribution levels at Suez and Ismailia sampling sites (A&B). Each column and vertical bar represents mean values and S.D. respectively.

Table 3: Total average PAH concentration levels in Red sea waters at sites from Suez and Ismailia governorates

Ring no.	Short name	S1	S2	I1	I2
PAH (ng/l)					
2-ring	Nap	388.0	88.5	299.1	55.8
		388.0	88.5	299.1	55.8
3-ring	Ace	321.0	95.4	277.3	88.5
	Fle	127.0	26.3	103.0	23.0
	Phe	199.0	39.8	207.0	20.3
	Ant	26.7	24.1	10.0	3.0
		673.7	185.6	597.3	134.8
4-ring	Frt	21.0	15.0	13.3	8.1
	Pyr	33.2	17.9	40.1	17.4
	BaA	210.3	10.5	38.7	6.1
	Chr	14.1	3.0	7.1	1.8
		278.6	46.4	99.2	33.4
5-ring	BbF	16.2	5.0	13.0	2.3
	BkF	9.4	2.1	3.2	1.2
	BaP	51.5	14.0	31.1	12.5
	DBA	0.0	0.7	0.0	0.0
		77.1	21.8	47.3	16.0
6-ring	BghiPe	29.4	2.1	14.5	2.6
	IDP	0.8	0.0	0.0	0.0
		30.2	2.1	14.5	2.6
	Total	1446.6	344.4	1057.4	242.5

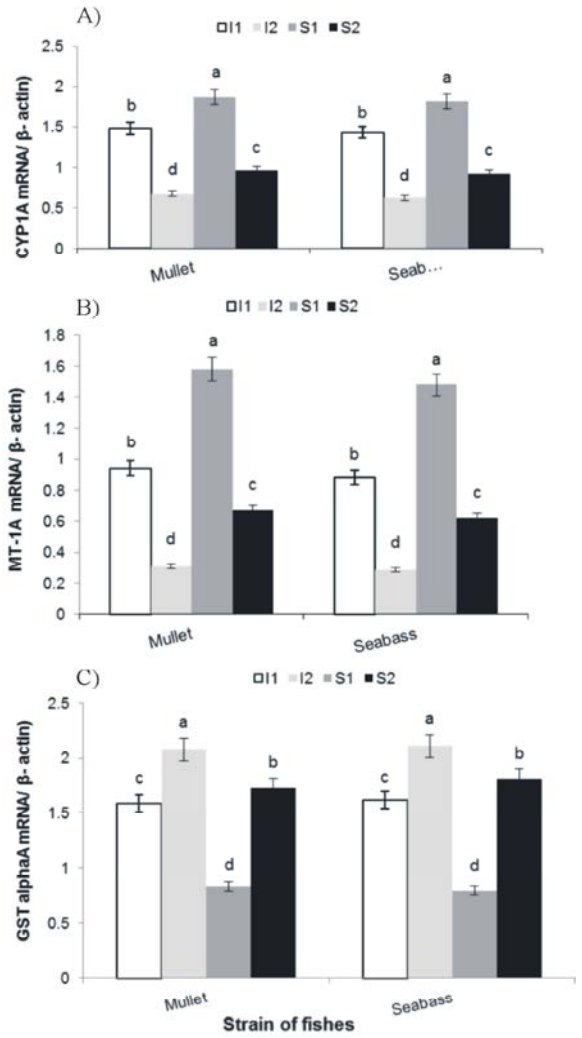

Fig. 3: The alterations of CYP1A (A), MT-1A (B) and GST alpha-mRNA (C) in liver tissues of Mullet and Sea-bass collected from I1 and I2 at Ismailia governorate and S1 and S2 at Suez gulf. Data are presented as mean±SEM.
Means with different letters, within locations, differ significantly (P = 0.05).

The PAH levels at all investigated sites in Suez Gulf showed that low molecular weight (LMW) (2-3 ring) PAHs (Nap, Ace, Fle and Phe) and medium molecular weight (MMW) (4ring) PAHs (Frt, Pyr, BaA and Chr) have higher concentrations over the high molecular weight (HMW) (5-6 ring) PAHs (Bb, BKF, Ba, DBA, Bghipe and IDP) (Fig. 2 B&C).

Expression of stress protein related genes (Cytochrome P450, CYP1A and metallothionein, MT-1A) and antioxidant enzyme (Glutathione-S-Transferase, GST alpha) in liver tissues of Mullet and Sea-bass collected from polluted locations in Ismailia governorate and Suez gulf is summarized in Figures 2 & 3. The results indicated a clear fact that expression levels of CYP1A, MT-A and GST alpha genes in the liver tissues of fish collected from polluted locations were altered significantly in comparison to the reference site (Fig. 2A). Nearly the same results were obtained when the quantitative real time-PCR assay was performed for each individual sample within each region throughout the sampling period.

The current results revealed a significant increase in the hepatic mRNA levels of CYP1A and MT-A genes in Mullet and Sea-bass collected from S1 location at Suez gulf compared with I1 location at Ismailia governorate and with the S2 and I2 locations as reference site (Fig. 3A&B). In addition, the expression levels of CYP1A and MT-A genes in Mullet and Sea-bass collected from Ismailia governorate were significantly higher than those within S2 and I2 locations as reference site (Fig. 3 B&C). In regard to GST alpha gene, fish collected from I1 location at Ismailia governorate and from S1 location at Suez gulf exhibited significantly lower expression levels than those in the fish collected from the reference sites (Fig. 3C). Moreover, the lowest expression level of antioxidant enzyme, GST alpha gene, was showed in Mullet and Sea-bass collected from S1 at Suez gulf compared to other locations. Additionally, the expression level of GST alpha gene was significantly low in Mullet and Sea-bass collected from Ismailia S1 governorate compared to the reference sites (Fig. 3C).

DISCUSSION

The average concentrations of PAHs were much higher at Suez sampling sites (S1 and S2) than those at Ismailia sampling sites (I1 and I2) as shown in Table 3. The significant higher levels at the S1 and I1 sites might be attributed to the presence of many petroleum ships that cross the canal every day and stop at the port might discharge some oils into the water plus to their incomplete fuel combustion emissions. Moreover, presence of many industrial facilities along the canal including petroleum and petroleum derivatives industries discharging their wastewater without treatment to the water [5].

The PAHs variation pattern might be attributed to the following factors; (i) the exhaust from motor ships and the atmospheric fallout as well can cause a significant accumulation of PAHs in the aquatic environment, where the meteorological conditions greatly influence fallout, particularly local fallout. (ii) The inputs of PAHs to water environment increase because of increasing the burning

for waste disposal. (iii) The warming activities, that emit PAHs especially in rural areas increase also in winter at low temperatures. (iv) PAH are easily decomposed at high temperatures and photo chemically degraded in the presence of sunlight in summer season [18].

The ring distribution of PAHs over all Suez and Ismailia sites showed that the LMW PAHs were the predominant. PAHs ring distribution followed the order of 3-ring > 2-ring > 4-ring > 5-ring > 6-ring. These results can be attributed to (i) the lower concentrations of HMW PAHs in the atmosphere and (ii) the lower solubility of HMW PAHs in water environment (Figs. 3A&B).

The sources of emission of PAHs in the sampling sites along Suez and Ismailia waters can be investigated from the average concentrations of PAH. Where at Suez sampling sites showed higher values at S1 site than S2 site, this can be due to the heavy oil discharge from the ships as well as the close of this site to the industrial areas around the Suez port, that may lead to huge amount of PAH emissions. Whereas, at Ismailia sites, I1 had higher PAH values over I2 site.

The morphological examination on the collected fish revealed no visible gross changes on the outside of fish collected from S1 location at Suez gulf and I1 location at Ismailia governorate. However, the weight of collected fish from Suez gulf was lower than those collected from Ismailia governorate.

In the current paper, additionally, we have focused our attention on some stress-related proteins such as MT-1A and CYP1A, whose mRNA resulted over expressed in liver of the Mullet and Sea-bass fish collected from the S1 location at Suez gulf and from I1 location at Ismailia governorate with high concentration of PAHs in Suez gulf compared to those collected from Ismailia governorate with low concentration of PAHs. Moreover, the mRNAs of GST in liver of the Mullet and Sea-bass fish collected from the S1 location at Suez gulf were higher than those from I1 location at Ismailia governorate. To avoid a permanent damage, it is useful to have biomarkers capable to diagnose a stress condition at its earliest onset to allow an immediate corrective intervention. The synthesis mRNAs coding for ‘‘stress-related proteins’’ is certainly a quite early ‘‘primary’’ response that can be easily detected by PCR once ‘‘proper’’ sequences are known [16].

The Cytochrome P450 constitute a multigene family of enzymes involved in the oxidation of many endogenous and xenobiotics substrates [25]. CYP1A, identified in most vertebrates, is widely used as biomarker when assessing exposure to contaminants in environmental system [16]. Also, in this case, we have observed an over expression of its messenger in the analyzed liver tissues of the fish collected from Suez gulf compared to those collected from Ismailia governorate and reference site. MTs are cysteine-rich metal binding proteins that play an essential role in the regulation of intracellular metal concentration [26]; moreover, it is known that the expression of its transcript, generally present at basal levels, is increased also after other physiological sub-lethal stressor [27]. Our experiments demonstrated that MT mRNA increases in liver tissues of the fish collected from Suez gulf compared to those collected from Ismailia governorate and reference site. Cytochrome P4501A (CYP1A) induction is a sensitive and specific adaptive response of organisms exposed to environmental pollutants, including PAHs (28). The CYP1A inducers, such as polychlorinated biphenyls (PCBs), chlorinated dibenzodioxins (PCDDs), dibenzofurans (PCDFs) and certain PAHs, act via the aryl hydrocarbon receptor (AHR), which is a ligand-activated transcription factor that mediates many of the biological effects of these compounds [28]. The CYP1A induction is used for assessing sublethal exposure to such pollutants [29]. Because CYP1A is not only at very low levels, expressed in the liver of fish in pristine environments [30], elevated levels are thus indicative of exposure to these pollutants and can be used as a biomarker of exposure [28, 31]. The CYP1A content and activity in the liver of fish living in or exposed to oil-polluted environments are related to the amounts of PAHs measured at polluted sites or in fish tissue [31]. In agreement with these finding, we have found that CYP1A was up-regulated in the and liver tissues of the fish collected from Suez gulf which are found with high concentration of PAHs compared to those with low concentration of PAHs in Ismailia governorate.

The present work indicated that the high concentration of PAHs was accompanied with decline in the mRNAs of GST in liver of the Mullet and Sea-bass fish collected from the S1 location at Suez gulf were higher than those from I1 location at Ismailia governorate. In agreements with our findings, Palanikumar *et al.* [32] reported that an inhibition of GST was observed in milkfish *Chanos chanos* after exposure to anthracene (one of the PAHs members). They indicated that GST was modulated due to oxidative stress of the anthracene. It is known that PAHs can generate ROS via production of PAH metabolites that include redox-cycling quinines and radical cations, the latter being associated with DNA damage [33]. Also, PAHs can damage mitochondrial DNA

and perturb mitochondrial function, alter gene expression which may enhance oxidative stress and decrease of antioxidant enzymes in these organelles [16]. Induction of stress related proteins including CYP1A via the AH receptor (AHR) by compounds such as chlorinated dioxins and PCBs can lead to ROS generation and oxidative damage [34], associated with the recalcitrance of these compounds to metabolism. Numerous PAHs also induce CYP1As via the AHR, but this induction has not been thought to generate ROS per se, due to the suitability of PAHs as CYP1A substrates. However, ROS generation may be enhanced in co-exposures to PAHs which include both AHR agonists and CYP1A inhibitors, exposures which give rise to synergistic cell toxicity [35, 36].

In conclusion, the current work suggests that the stress related genes and antioxidant enzyme genes studied in this paper represent valid biomarkers to detect variation of fish stress conditions attributed to PAHs.

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