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Correlation between Polyaromatic Hydrocarbons (PAHs) Discharges at El-Manzala Wetland with the Relative Gene Expression of *CYP19* Gene of the Nile Tilapia (*Oreochromis. niloticus*)

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ABSTRACT

El-Manzala Wetland is known to be of the most significant sources of inexpensive different fish species for human consumption in Egypt. The Lake receives pollution from various sources; domestic wastewater, industrial wastewater, and agricultural drainage directly without any treatments, which leads to many environmental disorders. The following research was conducted for lake water quality evaluation and how it impacts the expression of the aromatase (*CYP19a&b*) genes in *Oreochromis niloticus* tissues at different locations of El-Manzala Lake. Sampling was done during the summer of 2018 when water and fish samples were collected from five different locations of El-Manzala Lake. It was found that the studied locations recorded hazardous concentrations of polyaromatic hydrocarbons (PAHs) that may cause significant defects to aquatic organisms except in the reference location and affect the expression of *CYP19a&b* genes. The relative gene expressions of *CYP19*a in the ovary and the testis were measured and recorded in positive ranges, but revealed the controversial expression pattern of *CYP19*b gene in the brain tissues. Our results revealed that PAHs discharges at El-Manzala Lake are correlated with the gene expression of *CYP19* genes. The current findings suggested a correlation between biomarkers response and wastewater exposure as stressors. Besides, the assessed multiple general health indicators give prospective insights for Nile tilapia adaptive pattern and monitoring of pollution of freshwater ecosystems.

INTRODUCTION

Environmental pollution is a worldwide problem initiated by the progress of numerous industries, leading to the amended discharge of altered pollutants into ecosystems. Aquatic animals are exposed to atypical environmental conditions (**UNDP, (1997)**.

Polycyclic aromatic hydrocarbons, one of the leading chemical ingredients of petroleum, are widely used and are mostly surviving in almost all environmental matrices, as well as aquatic environments **(Medhat *et al.*, 2019)**. PAHs are lipophilic with larger campuses of less water-solubility and volatility. For these possessions, PAHs are instituted primarily in oily substances, sediment, soil, and too far less extent in water or air. Many PAHs are persistent, toxic, and carcinogenic compounds that can attack DNA by forming PAH-DNA adduct **(Xue and Warshawsky, 2005)**.

El-Manzala Lake is one of the most dynamic aquasystems that were revealed to many threats over the last six decades due to agricultural drainage, municipal and industrial wastewater affecting Lake Biodiversity greatly **(Elmorsi *et al.*, 2019)**. The average fish production of El-Manzala Lake is 60,000 tons annually, where tilapia signifies more than 65% of fish species in the Lake **(Abd El Tawab *et al.*, 2020)**.
Nile tilapia *(O. niloticus*) fish can repel poor environmental conditions and diseases owing to their physical power. Moreover, the necessities for their respiration are low, approving them to exist in low levels of oxygen and high ammonia levels **(Abdel-Shafy, 2002)**. It is a worldwide extremely adjustable cultured species **(Molnar *et al.*, 2008)**, reproduces continuously during the year, and its gonads are ripe all year round.

The study of variation in gene expression is crucial for many biological responses and understanding how organisms respond to environmental variations, such as different stressors **(Heid *et al.*, 1996)**. Whereas studies have mostly motivated the dynamics of gene expression of organisms under laboratory conditions, few studies have addressed the question of how organisms respond in an ecological context.

Under natural conditions, individuals are exposed to multiple and changing environmental indicators, thus providing an opportunity to determine gene expression patterns that cannot be exposed under laboratory experiments **(Whitehead and Crawford, 2006; Whitehead, 2012)**.

CYP enzymes have been used as bio-monitoring and indicative tools of pollution in the vertebrates, because there is a reliable increase in CYP enzyme levels upon exposure to PAHs **(Han *et al.*, 2014)**. CYP19 genes encrypted aromatase enzymes; it is the final step in estrogen biosynthesis, which works via stimulating the conversion of testosterone to estrogen. They are presented in cells that produce estrogen in the ovaries, placenta, testicles, adipose tissue, and brain **(Chang *et al.*, 2005)**. Two isoforms of CYP19 genes are expressed in teleost: *CYP19*a (gonadal aromatase) and *CYP1*9b (brain aromatase), which fuse to synthesize estrogens from androgens **(Cheshenko *et al.*, 2008)**. The reproductive concert is a vital feature in deducing the population dynamics of fish that have relatives with fish sustainability **(Ojaveer *et al.*, 2015)**.

The current study aims to describe the association between the level of *CYP19* gene expression of Nile tilapia with the different pollutant discharges at five localities of El-Manzala Lake (El-Gamil, El-Temsah, El-Bashtir, El-Serw, and El-Mataryia as a reference location) by determining pollution levels and perceiving the expression of gonadal (*CYP19*a) and brain (*CYP19*b) genes.

MATERIALS AND METHODS

Collection of samples

O. niloticus samples were attained from five different locations at El-Manzala Lake (ten adult fishes for each group). **Fig. 1** displays the locations of sample collection: **1.** El-Gamil**, 2.** El-serw, **3.** El-Bashtir, **4.** El-Temsah, and **5.** El-Matariya as a reference site. Simultaneously, samples of the studied fish were of a weight ranging from 80 to 100 grams for each group.

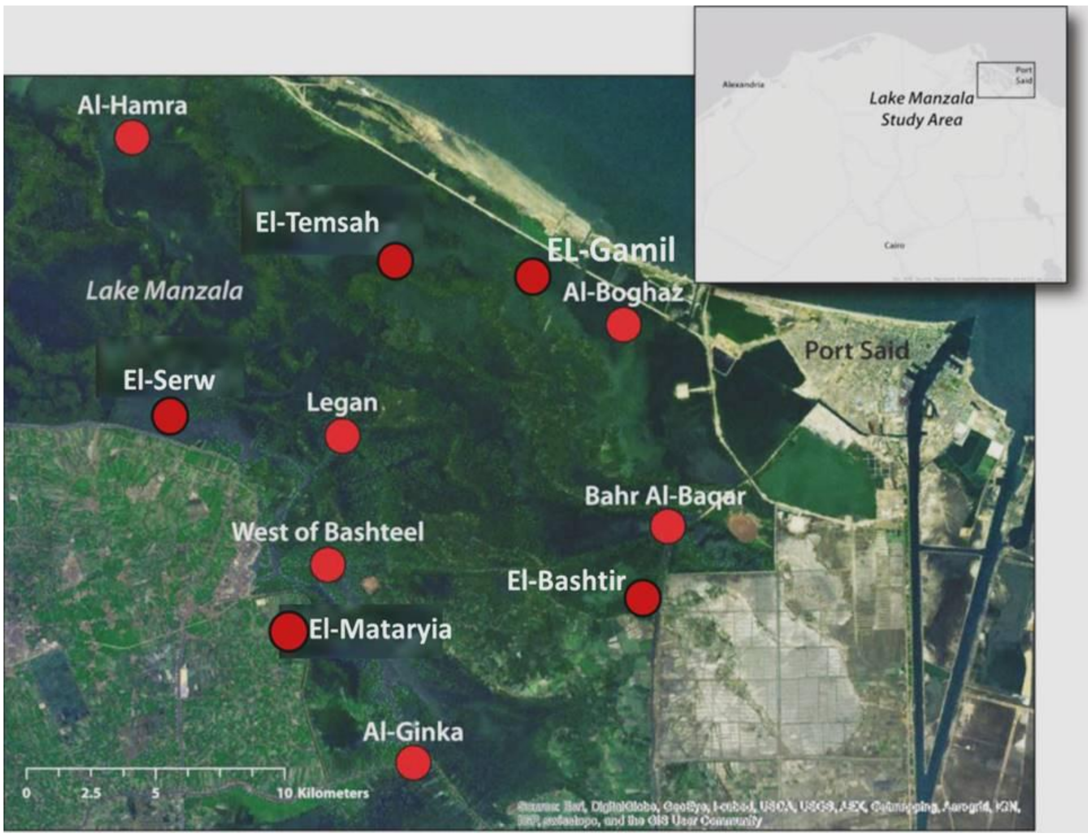

Fig. 1 Sampling locations from Lake El-Manzala **(El-Kady *et al.*, 2017)**

Extraction of the dissolved polycyclic aromatic hydrocarbons (PAHs) and determination of their levels in water

With the same conditions, the water sampled from the same studied locations that collected at 1 m depth of Lake Water by using a Niskin bottle.

. Samples were extracted in the area (three replicates from each group were studied), stored at 4 °C, and transferred to the lab for PAH analysis **(Parson *et al.*, 1985)**. PAHs were measured in water samples using a UVF-Spectrofluorometer (Sequoia-Turner

Model 450) at 360 nm and 415 nm, while the investigation was conducted as a chrysene unit **(APHA, 1989)**. Cleaning and fractionations were completed before the gas chromatography/flame ionization detector (GC/FID).

Total RNA extraction and cDNA synthesis

Samples from gonads (testes and ovaries) and brains of the females of *O.niloticus* were collected and immediately stored in liquid nitrogen. According to the manufacturer's instructions, RNA was extracted using Trizol Reagent (Invitrogen, UK). The RNA integrity was verified on agarose gel electrophoresis. The spectrophotometer (T80 UV/VIS) was used to determine the concentration and purity of the RNA. The absorbance at A_{260}/A_{280} represented the purity of RNA ranging from (1.8 to 2). According to the manufacturer's procedures, First-strand cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription kit (Qiagen, Germany).

Quantitative Real-Time PCR (QRT-PCR) assay

Specific primers were used for the target genes of Nile tilapia (*O. niloticus*). Primer sequences and references are mentioned in **Table 1**. Real-time qRT-PCR was carried out in Agilent Technologies Real-time PCR machine (Applied Biosystem 7500) using 2X PreMIX (SYBR Green with high ROX) (Enzynomics) to perform qRT-PCR with 1 µl cDNA (total 50 ng RNA) in a 20 µl reaction mixture. PCR conditions included 15 min at 95 °C, followed by 40 cycles at 95 °C, then at 60 °C for 15 s for each, and 72°C for 30 s. Melting curve analysis was performed at the end of each reaction to confirm specific PCR products. To determine the relative gene expression levels the $2^{-\Delta\Delta Ct}$ technique was used **(Livak and Schmittgen, 2001)**. Samples were tested in triplicate.

Table 1. Name, accession number, primer sequence, and product size for genes used for real-time PCR analysis

Target gene	Primer sequences	Product length	Accession number
Gonadal *CYP19*a	**F:**5-ACGCTGATACTGCTCGTCTG-3 **R:**5-GTAGTTGCTGGCTGTGCCTA-3	150	**U72071.1**
Brain *CYP19*b	**F:**5-GGAAACAGGAAGGCTACCCA-3 **R:**5-CTGACGCTCTATCAGCCACC-3	191	**NM_001279590.1**
β-actin	**F:**5-CCCAAAGCCAACAGGGAGAA-3 **R:**5-GTGGTGGTGAAGGAGTAGCC-3	275	**KJ126772.1**

Statistical analysis

All analyses were carried out by one-way Analysis of Variance (ANOVA) using the statistical program SPSS (version 16.0, software package. The Correlation between *CYP19* expression levels and PAHs in the water of El-Manzala Lake was evaluated using the Pearson correlation coefficient. In all tests, $p<0.05$ was considered a statistically significant difference. Data are presented as mean ± standard error of the mean.

RESULTS

PAHs analysis in water samples

Concentrations of 16 EPA (U.S. Environmental Protection Agency) priorities, are presented in **Table 2** as ppb on a dry weight (D.W). The highest value (409.1 ppb) was recorded at El-Gamil location, followed by 351.72 ppb at El-Bashtir, then 240.51 ppb at EL-Serw, while El-Temsah location recorded 94.73 ppb. The lowest value (3.46 ppb) was observed at the reference location (**Fig**. **2**).

Analysis of the concentrations of PAHs in water revealed that El-Gamil location had the highest values of some compounds such as naphthalene (139 ppb), acenaphthylene (0.63 ppb), acenaphthene (0.5 ppb), fluorene (0.46 ppb), phenanthrene (0.73 ppb), anthracene (2.27 ppb), fluoranthene (1.18 ppb), pyrene (13.09 ppb), dibenzo (a,h) anthracene (1.2 ppb), and Benzo (ghi) perylene (5.8 ppb). On the other hand, El-Bashtir location recorded the highest value in chrysene (340 ppb), benzo (a) pyrene (2.2 ppb), and indeno (1, 2,3cd) pyrene (1.3 ppb). However, the highest values of benzo (a) anthracene (4.19 ppb) and benzo (b) fluoranthene (5.07 ppb) were observed at El-Serw location.

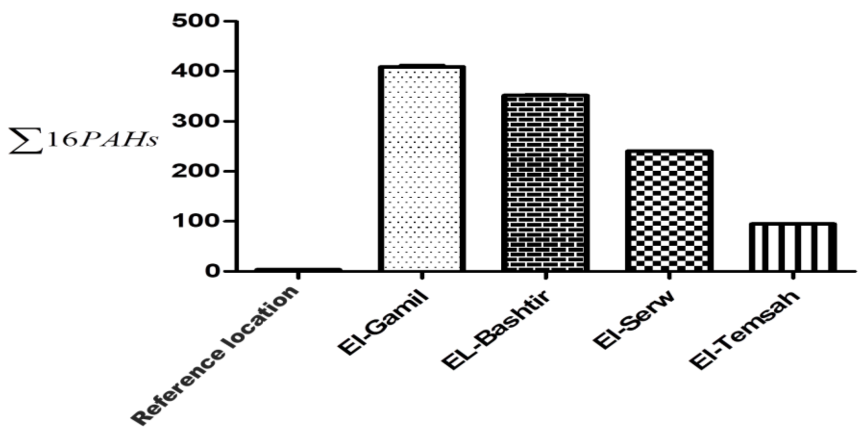

Fig. 2 Distribution of ∑16PAH in water samples at different locations of El Manzala Lake.

Table 2. Distribution of PAHs in tissue samples (ppb) collected from Lake El-Manzala, Egypt.

PAHs	Reference location	El-Gamil	El-Temsah	El-Bashtir	El-Serw
Naphthalene	0.1	139	ND	ND	ND
Acenaphthylene	ND	0.63	ND	0.15	ND
Acenaphthene	0.12	0.5	ND	ND	ND
Fluorene	ND	0.46	ND	ND	ND
Phenanthrene	0.05	0.73	0.09	0.07	ND
Anthracene	0.09	2.27	0.12	0.3	0.09
Fluoranthene	0.3	1.18	0.3	0.2	0.9
Pyrene	0.8	13.09	ND	ND	0.2
Benzo (a) anthracene	0.31	1.26	0.5	0.6	4.19
Chrysene	0.28	235.5	92.44	340	223.2
Benzo(b) fluoranthcene	ND	0.65	0.12	2.8	5.07
Benzo (k) fluoranthcne	1.2	4.01	0.2	0.9	5.6
Benzo (a) pyrene	0.1	1.7	0.06	2.2	1.26
Indeno (1, 2, 3 cd) pyrene	ND	1.1	0.1	1.3	ND
Dibenzo (a,h) anthracene	0.01	1.2	0.3	0.6	ND
Benzo (ghi) perylene	0.1	5.8	0.5	2.6	ND
∑ 16 PAHs	**3.46**	**409.08**	**94.73**	**351.72**	**240.51**

*ND: not determined

*Sum of the 16 priority PAHs as defined by US **EPA (2002)**.

Relative expression of gonadal *CYP19*a and brain *CYP19*b genes of *O. niloticus*

The transcription of the ovarian *CYP19*a gene recorded a significant value in the ovaries at El-Gamil location followed by El-Bashtir, El-Serw and El-Temsah locations **(Fig. 2A)** when these values were compared with the result of reference location (El-Mataryia) ($P < 0.05$). In addition, the overexpression of the ovarian *CYP19*a gene in the testes was recorded in the sequence: El-Gamil > El-Bashtir > El-Serw > El-Temsah with a significant value ($P < 0.05$) **(Fig. 2B).**

On the other hand, the brain *CYP19*b gene expression was controversial to that of gonadal *CYP19*a; the expression was significantly related to it at the reference location ($P < 0.01$). The highest expression at the reference location was followed by El-Temsah, El-Serw, El-Bashtir, and El-Gamil locations **(Fig. 2C).**

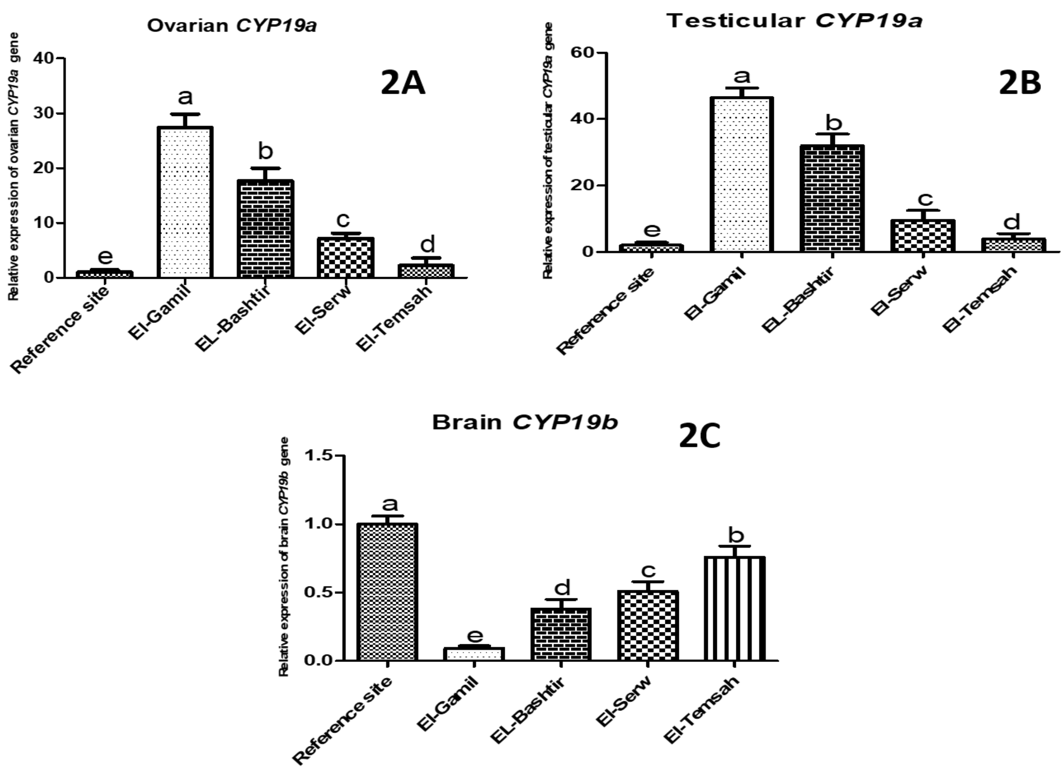

Fig. 2 Relative aromatase expression levels (aromatase: β-actin relatives) analyzed by quantitative PCR of individuals of Nile tilapia affected by different pollutions at the reference site, El-Gamil, El-Bashtir, El-Serw, and El-Temsah locations. Data are presented as mean ± SD. The diverse letters reveal significant variation. *CYP19*a in the ovary **(A)**, *CYP19*a in the testis **(B)**, and *CYP19*b in the brain of female **(C)**

Correlation between aromatase expression and PAHs at El-Manzala Lake:

We evidenced a correlation between the high ovarian aromatase (*CYP19a*) expression detected in the examined ovaries of Nile tilapia with elevated levels of PAHs that were measured in water with a significant value (*p-value*= 0.02). Also, we recorded a direct correlation between the same gene for the testes with PAHs with a substantial value (*p-value*= 0.04). However, the expression of *CYP19b* in the brain of females recorded an adverse correlation with PAHs with a significance (*p-value*= 0.019) as shown in **Table 3**.

Table 3. Pearson correlation between CYP19 expression and ∑PAHs

CYP19 expression	Correlation coefficient with ∑PAHs	p-value
CYP19a expression of the ovary	0.934*	0.020
CYP19a expression of the testis	0.893*	0.042
CYP19a expression of the brain of female	-0.936*	0.019

*Correlation of Pearson Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

Water pollution is considered one of the primary environmental health problems facing Egypt and the Middle East region **(Zulfahmi *et al.*, 2015)**. PAHs in the aquatic environment are of global concern due to their toxicity, hydrophobic properties, bio-accumulation, continuing inputs, and persistence. For instance, PAHs are known as dangerous pollutants, and there is a list of sixteen priority PAHs pollutants worldwide **(Paraso *et al.*, 2017)**. The (US EPA) has established 16 PAHs as the priority pollutants, 7 of which are potential human carcinogens **(Qin *et al.*, 2013)**.

In this context, the effect of different discharges of pollutants at El-Manzala Lake was detected by correlating the PAHs analysis with the relative gene expression of gonadal and brain *CYP19* genes. The existence of pollutant exposure results in reproductive hormone dysfunction, which influences the disruption of vitellogenin receptors in oocytes **(Livak and Schmittgen, 2001)**. Disturbance of vitellogenesis is often related to injury to liver tissue **(Muliari *et al.*, 2019)**. The effects of pollutants on fish reproduction have been studied by numerous researchers who confirmed adverse effects on fish organs and mainly their reproductive abilities **(Elmorsi *et al.*, 2019)**.

The results of this study revealed the up-regulation of the *CYP19*a gene in the gonads of Nile tilapia in different studied locations (El-Gamil, El-Bashtir, El-Serw, and El-Temsah) when they compared to El-Mataryia location (reference location), which lies in direct relation with pollution levels of PAHs. That is caused by different discharge resources of El-Manzala Lake. Meanwhile, the *CYP19*b gene expression in the brain tissues shows a controversial expression pattern to what we expect as it shows the highest expression pattern in the reference location detected upregulation of *cyp19a* mRNA levels in the gonad of *Rivulus marmoratus* and Atlantic salmon juveniles exposed to BPA (bisphenol A) and EE2 (17α-Ethinylestradiol) **(Lee *et al.*, 2006)**.

CYP family genes show a critical role in the immersion of the PAH. Also, the acceptance of the development of the fish CYP gene and its association with the metabolism of PAHs is important for the valuation and prediction of the ability of fish to acclimatize to xenobiotic risk **(Moses *et al.*, 1993)**, and it is induced by a broad range of xenobiotics such as PAHs **(Mansour *et al.*, 2018)**. PAHs are lethal substances to gonads as they can induce significant alterations in gene expression patterns.

The induced changes in gonadal tissues were also accompanied by the metabolism of arachidonic acid, androgen receptor to prostate-specific antigen signaling, and insulin-associated effects on lipogenesis **(Kawai *et al.*, 2012)**.

In support of this, the main sites of expression of CYP genes are the gonads and the brain. While it is also observed in other tissues such as the pituitary, retina, head kidney, kidney, spleen, fat, and liver that also show expression of one or both isoforms, which was proved in another previous study **(Denison and Nagy, 2003)**;

This may be caused by the plenty of discharged pollutants of complex composition from different sources of industrial, agricultural, and sewage facilities that recorded at El-Manzala Lake. Also, the present study revealed the inverse relationship linked the relative expression of *CYP19*b in the brain of females with the pollution at El-Temsah, El-Serw, El-Bashtir, and El-Gamil that showed the maximum levels at both clean and recovered areas.

Meanwhile, the expression was down-regulated when the levels of pollution increased, and these results may be attributed to the estrogen-related disorders leading to gradual loss of reproductive function after prolonged exposure to pollution **(Colli-Dula *et al.*, 2018)**. Others discussed the impacts of different environmental stresses on the ovarian aromatase (*CYP19*a) in Nile tilapia, such as the studying the effect of salinity as environmental stress to find that the expression of *CYP19*a in the gonads of *O. niloticus* as a response to the stress of salinity revealed the positive relation between salinity and the relative CYP19a expression in the ovary and testis of all treatments with marked higher values in ovary than testis **(Piferrer and Bla'zquez, 2006)**. The same findings were reported, and they mentioned the small brain aromatase activity in female perch at the leachate-contaminated Molnbyggen Lake **(Guyón *et al.*, 2012)**.

Conversely to our results, higher levels of *CYP19*b gene expression in the brains of killifish were observed in the highly contaminated than uncontaminated environments **(Abu Almaaty *et al.*, 2019)**. Also, an increase in brain aromatase activity was observed in fish species collected in rivers that receive wastewater treatment plants **(Noaksson *et al.*, 2003)**. Elevated activities of brain aromatase in mosquito fish living downstream of a pollution source in paper mill effluents were reported **(Greytak *et al.*, 2005)**.

In the present study, the knockdown factor is a critical point for gene expression of aromatase because it is an indicator of the reduction of gene expression in the different sites with an adverse proportion to the expression of the different genes, for the relative expression of *CYP19*a in the ovary, El-Temsah site (the only site which had mRNA knockdown of 77.6%).

The expression of *CYP19*a in the testis was higher than in the ovary at all sites that we recorded their knockdown factors in the different sites with negative values. The relative expression of *CYP19*b in the brain of females had mRNA knockdown at all the studied sites starting with the highest value (90.75%) at El-Gamil site, 62.13% at El-Bashtir site, 48.38% at El-Serw site, ending with the lowest percentage at El-Temsah site (33.55%).

Field monitoring has revealed a correlation between PAHs concentrations in the environment (or metabolites in individuals) whatever PAHs origin, and reproductive defects in fish **(Richardson and Ternes, 2014; Lee *et al*., 2013)**. This kind of monitoring analysis is essential for the environment but biomarkers often only allow raw estimates of disruptions and whatever actual reproduction output cannot be measured. Effects on population are therefore often only reached through modeling approaches **(GAFRD, 2014)**.

However, there was a limitation of this study. PAH did not exist separately in the aquatic ecosystems; thus, other environmental stressors might contribute to the responses of reproductive toxicity with PAHs, notwithstanding its limitation; this study did suggest the correlation of the reproductive toxicity responses with the degree of PAH-contaminated areas.

CONCLUSION

Our results indicated that *CYP19* gene expression seems to be induced by PAHs pollution, therefore, the appropriateness of CYP19 as a marker of pollution exposure in fish may be confident under field conditions. The results proved that the PAHs accumulation in water may vary depending on changes in the environmental conditions. The relative expressions of *CYP19*a in ovaries and testes are directly correlated with PAHs pollution levels. At the same time, a negative correlation was detected between the relative expression of *CYP19*b in the brain of females and PAHs. Therefore, further laboratory studies are needed to evaluate the impacts of these pollutants on the endocrine disruption of fish.

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