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Effect of polyaromatic hydrocarbons on cellular cytochrome P450 1A induction

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ABSTRACT

The detection of cytochrome P450 1A in *S. rivulatus* liver and gills was used as a biomarker for the exposure to polyaromatic hydrocarbons (PAHs) as organic pollutants along the Bay of Alexandria, Egypt. PAHs were determined in both sediment and S. *rivulatus* tissues in Abu Qir Bay, Alexandria as a test area and the Matrouh coast as a control area. High levels of PAHs in Abu Qir Bay area and ratios of specific PAH compounds indicate the petrogenic origin of hydrocarbons. Both antioxidant activity and oxidative stress markers increased significantly. The protein expression of P450 1A in the liver and gill of S. *rivulatus* was markedly induced. Moreover, the toxicity in fish organs was supported by histopathological observations. These data suggest correlation between bioaccumulated PAH and P450 1A induction and provide strong evidence of the importance of P450 1A as a biomarker of the exposure of S. *rivulatus* to xenobiotics.

Descriptors: Aromatic hydrocarbons, Cytochrome P450, *Siganus rivulatus*, Antioxidants, Western blotting.

INTRODUCTION

Aquatic pollution is one of the greatest negative legacies of 20th-century growth (Laranjeiro et al. 2015). Due to a large number of industrial, agricultural, domestic waste, effluents, as well as hazardous substances, environmental pollution has increased significantly in recent decades (Bouchet et al. 2012). Chemical pollutants such as polycyclic aromatic hydrocarbons (PAHs) can concentrate in tissues of aquatic organisms; concentrations of chemical pollutants in tissues may be of community health concern for both animals and humans (Honda et al. 2020; Juma et al. 2018). Contaminants accumulated in fish tissues can cause reactions that produce reactive oxygen species (ROS) resulting in oxidative stress, (Lushchak, 2016). There are many studies on antioxidant enzymes such as glutathione-S-transferase (GST) and catalase (CAT) because they provide an early warning of contamination in animals. Antioxidant enzymes can be found in almost all vertebrate tissues, with activity especially in the liver, which is a key organ in the transformation of ROS. These enzymes have the potential to be used as biomarkers to detect harmful health impacts early in their development (An et al. 2008). Changes in the action of antioxidant enzymes, damaged DNA bases, the result of protein oxidation and lipid peroxidation products are all examples of oxidative stress (Sarker et al. 2018). In addition, PAHs may cause the most commonly used biochemical indicator in environmental pollutant biomonitoring to be cytochrome P450 1A (Ikue et al. 2016). It is involved

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in the metabolism of xenobiotics and drugs (Parente et al.2008). Planar aromatic hydrocarbons can cause the induction of cytochrome P450 1A proteins via an intracellular aryl hydrocarbon receptor (AhR) (Poland and Knutson 1982), located mainly in the liver but also present in extrahepatic tissues (Yusuf et al. 2015). According to the high binding of the ligand TCDD, the receptor has translocated to the nucleus where it can dimerize with an AhR nuclear translocator (Petrulis JR and Perdew 2002; Backlund and Ingelman 2005). The present research aims to estimate the impact of pollution from the atmosphere on biomarkers of oxidative stress in *S. rivulatus* fishes and discuss the role that this biomonitoring could assign, as well as create a baseline for biomarker research in Alexandria Abu Qir Bay, Egypt. In addition, it aims to demonstrate the oxidative stress response using an antioxidant enzyme system and lipid peroxidation agent obtained from the contaminated area (Abu Qir Bay) in both the liver and gill tissues of *S. rivulatus*. It also aims to investigate the role of gill and liver cytochrome activation at CYP1A in regulating biological effects. As a result, biomarkers of contact with PAHs and related organic contaminants in the Bay of Alexandria include immunochemical detection of cytochrome P450 1A in *S. rivulatus* liver and gills. The uniqueness of this manuscript is that it is a form of aquatic pollution monitoring in which the investigation is based on industrial and agricultural waste contamination in specific areas of the Mediterranean Sea. The target study was conducted during a popular fishing season that is economically accessible to a large number of people.

METHODS

Materials

Chemicals used were obtained from Merck and Sigma Aldrich. The antibodies are from Boster Biological Technology (Anti-Cytochrome P450 2D6/CYP2D6 Picoband™ Antibody primary antibody against fish CYPIA (Human IgG polyclonal antibody for Cytochrome P450 2D6 (CYP2D6) detection, Rabbit Anti-Human IgG (H+L) Secondary Antibody, HRP Conjugate). All solvents were pesticide grade and purchased from Merck.

Chemical analysis

S. rivulatus morphological structures were determined in both the test area (Abu Qir Bay) and the control area (Matrouh coast). Weight and length of *S. rivulatus* collected from both test and control areas were measured, as was the hepato-somatic index (HIS, liver weight/bodyweight x 100) of fish (Jangaard et al. 1967). The chemical analysis determined chemical xenobiotics in sediment samples collected during July 2017 from ten stations, distributed along Abu Qir Bay, Alexandria and from ten stations scattered along the Matrouh coast. The determination of chemical xenobiotics was performed by the determination of hydrocarbons in both sediment and *S. rivulatus* tissues by using a gas chromatography (GC) flame ionization detector as previously described (Abdallah 2017). Six tests on hydrocarbon reference materials were performed to ensure the analytical reliability and recovery performance as well as the accuracy of the findings. NRC-IMB of Canada provided HS-5 (sediment), and NIST of the United States provided SRM 2974 (freeze-dried mussel tissue; *Mytilus edulis*). Sediment samples of known hydrocarbon levels spiked with a mixture of 2 g each of hydrocarbons were analyzed as above to validate the analytical method used in the present study. The laboratory results showed recovery efficiency ranged from 90-110%, 85-97%, and 94-102% for HS-5, SRM-2974, and the spiked samples, respectively.

Biochemical assays

The biochemical study for liver and gill tissues of *S. rivulatus* collected from both control and test area included the determination of the following parameters: (GST) activity, reduced glutathione (GSH) level, CAT activity, and lipid peroxidation. GST specific activity was measured by the reaction of 1-chloro-2, 4-dinitrobenzene with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm (Khan et al. 1992) and the activity of GST was performed by observing it as nmol/min/mg protein. The determination of GSH was performed by its reducing effect on 5, 5- dithiobis (2-nitrobenzoic acid) to give a yellow compound that is measured at 405 nm and expressed as nmol/mg protein (Beutler 1963). CAT activity was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm, and the activity was expressed as nmol/min/mg

protein (Aebi 1984). At the same time, the determination of lipid peroxidation was done by measuring the reaction of malondialdehyde (a breakdown product of lipid peroxides) with thiobarbituric acid. The resulting thiobarbituric acid reacting substances (TBARS) level was measured at 535 nm (Buege and Aust 1978). Lipid peroxidation was expressed as TBARS concentration nmol/mg protein. Total proteins were assayed by measuring the purple biuret complex (Doumas et al., 1997).

SDS-PAGE and Western blot analysis

Liver and gill tissue samples were homogenized with tissue extraction reagent (Lysis buffer for protein extraction from tissue (Invitrogen FNN0071)) supplemented with complete cocktails protease inhibitors (Roche, USA) (Towbin et al. 1979). The primary antibodies were diluted at 1: 1000. The immune blots were performed on the prepared total cell extracts as previously described and incubated with the HRP-conjugated anti-rabbit secondary antibody at a 1: 5000 dilution. The immune-reactive bands were detected with a detection kit (Ultra TMB-Blotting Solution, Thermo Fisher Scientific Inc., USA). The membranes were photographed, and the densitometry of bands was performed by using Quantity One Analysis Software (Bio-Rad, USA) (Towbin et al. 1979).

Histological preparation of liver and gill tissues

The tissue-fixed sections in 10% formalin were used for histopathological examination in the histopathology laboratory of the Faculty of Medicine, Alexandria University. Small blocks of liver tissues were removed and placed as soon as possible into glutaraldehyde-formalin fixative buffered with 0.1M sodium phosphate buffer (pH=7.4) for around 1 hour. After two runs in the buffer for 4 h, fixed samples were post-fixed in 1% buffered osmium tetroxide (OsO4) for 1-2 h. The tissue pieces were subsequently washed twice in the same buffer for 30 min. The specimens were then dehydrated and polymerized. A rotary microtome was used to cut 5 µm stained sections from the paraffin-embedded block and stained with hematoxylin and eosin (H&E) stain for the examination of any histopathological changes under the light microscope. Ultrathin sections were double-stained with freshly prepared uranyl acetate (Reynolds 1963) for 20 minutes and lead citrate for 5 minutes. Scoping grids were achieved by using JEOL 100CX transmission electron microscope of the Faculty of Science, Alexandria University.

Statistical analysis

Data were presented as a mean ± standard error (SE). The difference between the groups was assessed using one-way ANOVA and LSD post hoc test. A value of $P < 0.05$ was accepted as significant.

RESULTS

The morphological results showed that the range of weight and length for *S. rivulatus* collected from each region was similar. The hepatosomatic index for *S. rivulatus* collected from Abu Qir Bay was 1.83-fold greater than those obtained from the coast of Matrouh (Figure 1).

For xenobiotics, the results of the determination of hydrocarbons by GC analysis in both sediment and *S. rivulatus* tissues are shown in detail in the supporting file S1. The range of concentrations of total hydrocarbons in sediment samples collected from the Matrouh coast waa 272.78 - 391.26 ng /g DW, as opposed to 1928.5 - 2749.96 ng /g DW in sediment

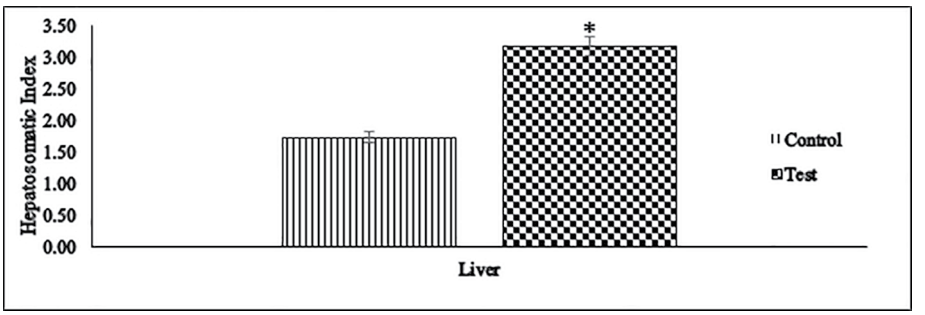

Figure 1. Hepatosomatic index of *S. rivulatus* collected from Abu Qir Bay (test area) compared to that collected from the Matrouh coast (control area). Data (n = 10) are expressed as mean ± SE. *p<0.05 (compared to control).

samples collected from Abu Qir Bay. The range of total concentration of hydrocarbons in *S. rivulatus* gill collected from the Matrouh coast was 65.3 - 102.1 ng/gDW and 1240.5 - 2025.4 ng/gDW in Abu Qir Bay. The bay sediments had PAH concentrations of 445 - 2660 g/kg DW. Overall, concentrations in mussels were higher than their corresponding sediment concentrations, indicating their high bioavailability (242 - 3880 g/kg DW). The PAH distribution pattern was similar for mussels and sediments, especially for high-contamination sediments, and they were dominated by high molecular weight PAHs (4 - 6-rings) (Figure 2).

For biomarkers, the specific activity of GST in the liver and gill of *S. rivulatus* collected from Abu Qir Bay increased by 68.03% and 118.9% respectively, relative to those collected from the coast of Matrouh. Additionally, the level of liver and gill GSH for *S. rivulatus* collected from Abu Qir Bay increased by 89.3% and 104.8% respectively, compared to those collected from the coast of Matrouh. The difference compared to the control areas were significant. CAT specific activity in the liver and gill of *S. rivulatus* collected from Abu Qir Bay increased by 170.8% and 17.7% respectively, compared to those collected from the coast of Matrouh. The lipid peroxidation level for liver and gill of *S. rivulatus* collected from Abu Qir Bay increased significantly by 309.8 and 230.8%, respectively, compared to those collected from the coast of Matrouh ($p<0.5$).

Western blotting analysis indicated that cytochrome P450 1A antibody was immunoreactive with a protein of a molecular weight of 45 KD extracted from liver and gill of *S. rivulatus* collected from control and test areas. The findings showed that exposure to chemical contaminants in the test area induced the expression of cytochrome P450 1A protein in the liver and gill (Figure 3). Also, the protein synthesis of cytochrome P450 1A tended to be hampered by the concentration of chemical pollutants in fish liver and gill obtained from the test region (Figure 3). The relative optical density using the ImageJ software system shows that the expression of cytochrome P450 1A protein in the liver and gill is significantly up-regulated in *S. rivulatus* collected from chemical pollutant-exposed test area (Figure 4). The exposure to chemical contaminants in the test region caused cytochrome P450 1A protein production in the liver and gill.

The results of the histopathological studies for liver and gill tissues were illustrated in Figure 5. Mononucleated hepatocytes, electron-dense sinusoidal space (S), and spherical central nucleus (N) with the normal nuclear envelope (Ne) were viewed for histopathological analysis. Centric large nuclei and exocentric nuclei were found, and some nuclei revealed exocentric nuclei (Figure 5A). Hepatocytes, had a normal nuclear shell and a small amount of concentrated heterochromatin attached to the nucleolus (Nu). Also, rough endoplasmic reticulum (RER), spherical and lacking mitochondria (M) with thin tubular cristae, and a small number of lipid droplets (L) (Figure 5A) were seen. Mononucleated hepatocytes, with a spherical central nucleus (N), with normal nuclear envelope (NE), and some with large centric nuclei were found. The nucleus of Hepatocyte nuclei had mostly a normal nuclear shell, a small amount of condensed heterochromatin attached to the nucleolus (NU), spherical and avoiding mitochondria (M) with thin tubular cristae, a limited number of lipid droplets (L), and glycogen (G) (Figure 5B). The hepatocyte core contains masses of Kupffer cell (K), mononucleated hepatocytes, and a spherical

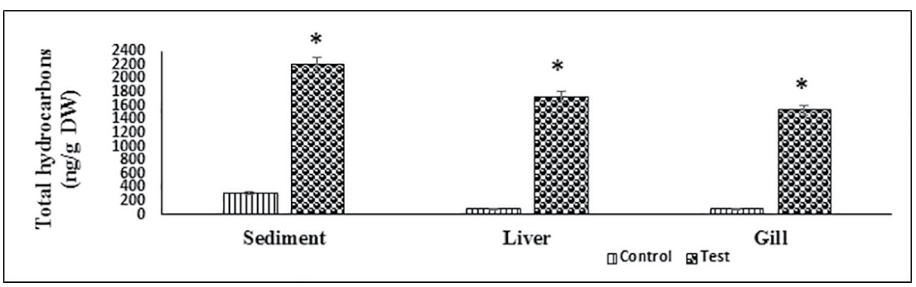

Figure 2. Total hydrocarbon concentrations (ng/g DW) in sediment, liver, and gills of *S. Rivulatus* samples collected from Abu Qir Bay (test area) comparing to samples collected from Matrouh coast (control area). Data are expressed as mean ± SE. *$p<0.05$ (compared to control).

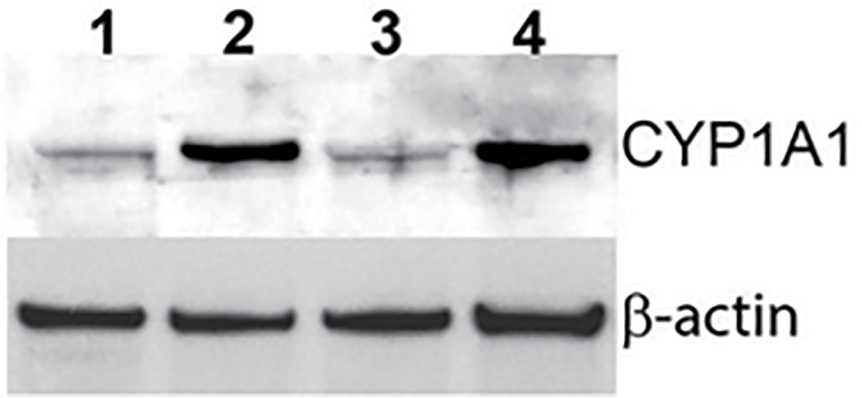

Figure 3. Representative immunoblots of cytochrome P450 1A for gill control (1), gill test (2), liver control (3), and liver test (4) pooled from groups of ten fishes. β-Actin was probed with antibodies to cytochrome P450 1A.

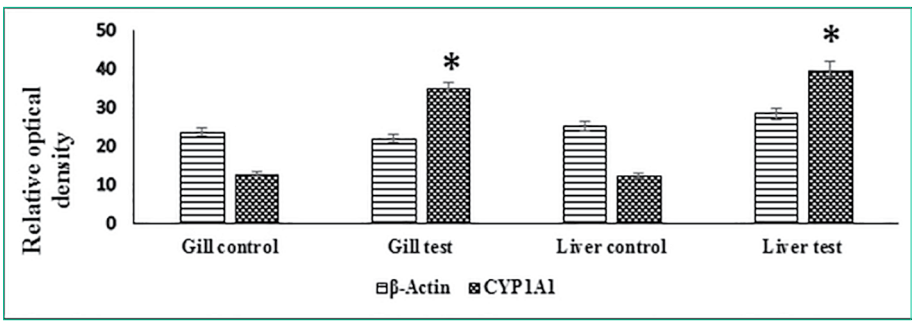

Figure 4. Densitometric analysis of protein levels as densitometry units (DU) of band intensity. Data (n = 10) are expressed as mean ± SE. *p<0.05 (compared to control).

nucleus (N). The nucleus (N), with the irregular nuclear envelope (NE), Kuffer cell (K), swollen mitochondria (M), a large number of microbodies (Mi), numerous primary lysosomes (LY), peroxisomes (P), large empty vacuoles (V) with flocculent materials and myline, and glycogen (G), was different compared to controls (Figure 5, panel E.) Also, a large Melan macrophage core (MC) was noted, with various primary and secondary lysosomes (Ly) varying in size and stainability, a significant number of peroxisomes (P), various lipid droplets (L), and a large amount of glycogen-housed cytoplasm (Figure 5F). In Figure 5, panel G has an altered nucleus (N), various primary and secondary lysosomes (Ly) varying in size and stainability, swollen mitochondria (M), large numbers of peroxisomes (P), various lipid droplets (L), and large numbers of microbodies (MI). The cytoplasm contains large amounts of glycogen (G). The electron micrograph of the gill epithelium (Figure 5, panels H, I, J, K, L, M & N) also exhibited similar observations.

Discussion

The use of biomarkers measured at the molecular and cellular level in environmental quality assessment is relevant as a sensitive 'early warning' tool for biological effect measurement. This study highlights the significance of such biomarkers concerning their use and current limitations. Our findings agree with Ibor et al. (2017), who showed a significant increase in biotransformation systems in phases I and II and oxidative stress associated with metals and PAHs in male and female fish from Eleyele Lake when compared to a reference site. The findings of the current study also agree with an earlier study detecting PAH concentration in *Scarus niger* collected from the Xisha Islands (test area) and around twice as high as that of *Scarus niger* collected from the Nansha Islands (control zone) (Li et al. 2019). The dominant compounds were found as 2-ring and 3-ring PAHs. The main

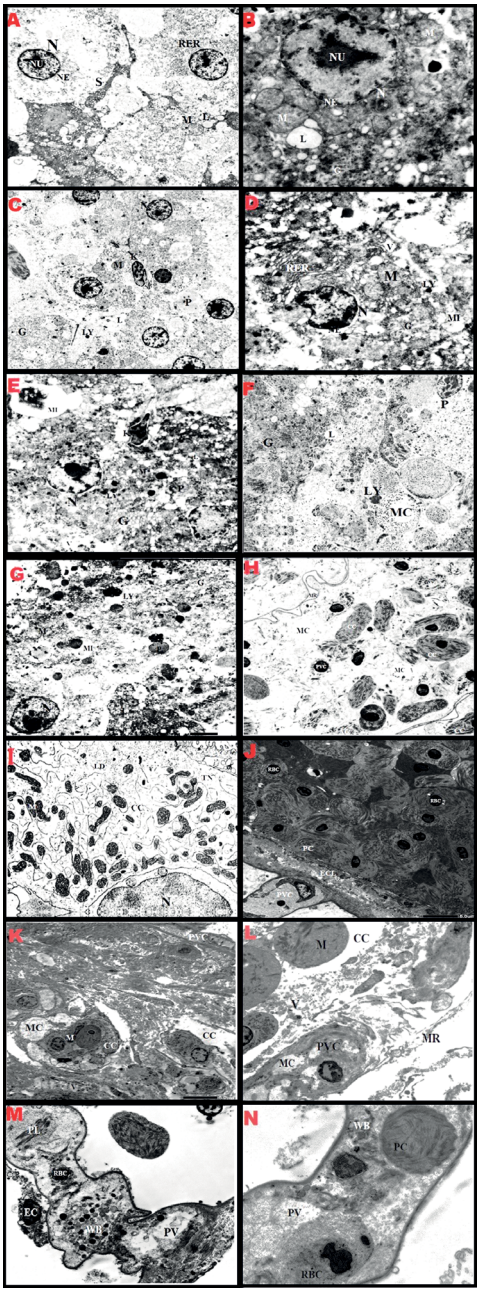

Figure 5. Electron micrograph of the liver (A - G) and gill epithelium (H - N) of *S. rivulatus* collected from the Matrouh coast (control area). Panels A - N: Glutaraldehyde fixed- OsO4 post fixed- uranyl acetate - lead citrate stained preparation. A: X, 6000; B: X, 4000; C - G: X, 4500; D: X, 4500; E: X, 5500; F: X, 6500; G: X, 5500; H: X, 6000; I: X, 8000; J: X, 5000; K: X, 6000; L: X, 5000; M: X, 4000; N: X, 5000.

sources of PAH were coal and biomass combustion (50.4%), petroleum sources (25.9%), and vehicle emissions (16.1%), based on qualitative and quantitative analyses.

By applying different PAH ratios, PAHs were found to derive mainly from pyrogenic sources, either from the combustion of grass, wood, and coal (most samples) or from the combustion of petroleum (harbor area). The output of a Screening Level Ecological Risk Assessment (SLERA) on the bay sediments revealed that adverse ecological effects on benthic organisms are expected to occur in only one sample. Therefore, PAHs in Abu Qir Bay are not considered to be of concern for contamination. Adverse health consequences are also not expected to arise from Abu Qir Bay intake of examined mussels linked to PAHs. PAHs in water and fish samples were slightly different, but correlation analysis showed that the PAHs came from the same source.

Using a complex suite of markers inside a single target tissue may often produce findings that are hard to reproduce in experimental assays. For example, the differences in liver antioxidant parameters were investigated in brown bullhead fish in a PAH-contaminated site and a control site in Ohio, USA and no between-site variations were observed in the levels of glutathione reductase, glutathione S-transferase, and glutathione peroxidase (Myers et al. 2000). However, other antioxidant parameters such as superoxide dismutase (SOD), catalase, and total glutathione levels in tissues tended to correlate with environmental exposure to PAHs. In another report, 11 biochemical markers of aquatic pollutants in the livers of chub captured at several river sampling sites were evaluated, finding multiple contaminants (Machala et al. 2001). The biochemical markers of oxidative stress, including *in vivo* lipid peroxidation and *in vitro* ROS production, did not correlate with contaminant concentrations, while glutathione-dependent enzymes formed an appropriate battery of biomarkers of exposure (Machala et al. 2001). These studies are among many that illustrate the complexities associated with drawing meaningful conclusions from the field-observed differential antioxidant responses.

Moreover, the findings of the present study are in accordance with those who have tested the same set of biomarkers to categorize the safety of the Tamar estuary in the UK (Shaw et al. 2011). Shaw et al. (2011) classified stress using gene expression analysis based on microarray assays and found that lysosomal stability and oxidative-stress markers were associated with PAH concentrations in the water, and the number of genes with altered transcription patterns at most polluted

sites was highest. However, no changes were observed in the neutral lipids with contamination levels (Shaw et al. 2011). Hence, PAHs may act as ligands for AhR, a subunit of a ligand-activated transcription factor that regulates a variety of cellular activities, including the stimulation of xenobiotic metabolism enzymes (Ibor et al. 2017; Zhou et al. 2010). When PAHs bind to the AhR in the cytosol, the molecules are nuclear translocated, the aryl hydrocarbon receptor nuclear translocator (ARNT) is attached, and complex molecules capable of interacting with large macromolecules are produced (Zhou et al. 2010; Chiaro et al. 2007). These interactions change the gene expression and contribute to the up-regulation of the enzyme CYP450 targeting PAHs and inducing their biotransformation (Santos et al. 2018). CYP-mediated epoxidation is always the first step in PAH biotransformation and starts in the endoplasmic reticulum. More hydroxylation in the nucleophilic attack forming adducts of DNA produces active metabolites (i.e., PAH-diols) (Pampanin et al. 2016; Pampanin et al. 2017; Pirsaheb et al. 2018).

Peroxidases and certain CYP450 enzymes may also catalyze PAHs with one-electron oxidation, producing toxic radicals that can be further oxidized to quinone radicals, which can cause oxidative stress and high cytotoxicity levels (Ibor et al. 2017, Verma et al. 2012). However, Phase II metabolism of intermediate metabolites is conducted by enzymes such as glutathione-S-transferases, UDP glucuronyl transferase, and sulfotransferases, promoting metabolic clearance and excretion of PAH-metabolites (Santos et al. 2018, Lüchmann et al. 2014). Also, hatchery-reared turbot (*Scophthalmus maximus L*) was exposed to sediment obtained from contaminated sites in Cork Harbour, Ireland as a reference site (Hartl et al. 2007). CYP studies in *A. multispinnis* showed that AhR-ligand was induced as 3-MC (Verma et al. 2012). It correlates with other research in fish such as 3-methylcholanthrene (3MC) and BNF (Goksøyr et al. 1992; Stegeman and Hahn 1994) on the activation of AhR-binding ligands for hepatic CYP1A. The total concentration of P4501A is widely used, is very simple to measure, and can be a contamination screening tool, especially for PAH and planar polychlorinated biphenyls.

In conclusion, Abu Qir Bay is heavily contaminated by polyaromatic hydrocarbons. The present investigation suggests that biomarkers of oxidative stress, in particular the estimation of antioxidant systems in fish, provide a useful indicator of marine ecosystem pollution. In particular, the correlation between bioaccumulated PAHs and cytochrome P4501A induction provided strong evidence of the utility of such biomarker of xenobiotic exposure in *S. rivulatus*.

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