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Physiological and Histopathological Alterations Induced by Phenanthrene on Marine Cultured Tilapia Fish *Oreochromis spilurus saudii*

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Abstract. The aim of this study was to investigate the effects of different concentrations of phenanthrene (0, 10, 25 50 mg L^{-1}) mixed with spike food on four treatment groups of 20 marine cultured tilapia. Such effects include morphological, structural, functional, and genetic changes. Significant reductions ($P<0.05$) in fish length and weight (7.09 cm and 6.53 g) were found in the group treated with the highest dose of phenanthrene (50 mg L^{-1}). The fluorescence spectrophotometry analysis of treated fish oil indicated a positive correlation between the phenanthrene concentrations and the dosage of phenanthrene administered to the fish. Moreover, histological changes in liver tissue along with genetic changes also escalated as the concentration of phenanthrene increased. They were. The results revealed that phenanthrene was present in the biological system of the fish, and its effects on fish tissues were confirmed. Further studies are recommended to investigate the toxicity of phenanthrene on fishes and its biomarkers at biochemical levels.

Keywords: Marine tilapia, *Oreochromis spilurus*, PAH's, Phenanthrene, Fluorescence.

1. Introduction

Saudi Arabia's unique geographical location provides access to two major bodies of water in the Middle East (*i.e.* the Arabic Gulf and the Red Sea), which allows for the shipment of oil and petrochemical secondary products overseas from multiple ports[1]. Saudi Arabia's continuous production and shipment of enormous amounts of oil and petrochemical products increases the possibility of releasing crude oil and petrochemical by-products into open seas[2]. Marine organisms and wildlife are subjected to these pollutants, which may cause intensive disruption of the ecosystem, thereby decreasing biodiversity and biota in general[3]. It is well known that crude oil is a chemical mixture of approximately 17,000 hydrocarbon compounds[4]. The largest group in this mixture are referred to as polycyclic aromatic hydrocarbons (PAHs).

PAHs are a group of chemicals produced by partially burning organic materials, including coal, oil, gas, wood and coil. Polycyclic aromatic hydrocarbons (PAHs) are present in composite mixtures. Among the numerous PAH compounds, phenanthrene, naphthalene, chrysene and pyrene are the most abundant pollutants with an organic root[5]. They are primarily derived from pyrogenic and petrogenic sources. Given the pervasive use of petroleum products, PAH contaminants can also be found in water, soil and air near contaminated areas, factories,

oil production sites and transportation lines. The investigation of PAH toxicity, physiological effects and genotoxic potential in marine organisms has become a very useful tool in the assessment of pollution and its effect on environmental conditions[6]. Recently, PAHs have received significant attention from researchers due to the carcinogenic and mutagenic effects of these compounds[7].

Fig. 1. Chemical structure of Phenanthrene.

Phenanthrene (Phe) is a PAH constituent with a relatively low molecular weight of 178.22 g/mol. It is composed of three fused benzene rings (Fig.1), and it is found as a colourless crystalline solid primarily derived from pyrogenic sources[5,8]. It is widely distributed in aquatic environments and has been found in surface water, tap water, wastewater and dry lake residues. It is also found in seafood that is collected from polluted water, including numerous fish species. Ultimately, it indirectly causes chronic diseases in humans by consuming polluted foods[9].

Tilapia fish is endemic to Africa that belongs to the cichlid group. This group comprises three main genera: *Oreochromis*, *Sarotherodon* and *Tilapia*. All species of tilapia are nest builders, and after fertilization, the eggs are secured in the nest by the parents. Immediately thereafter, the fertilized eggs are collected by the parents in their mouths for incubation and held for several days until hatching[10].

Farmers in tropical and sub-tropical regions of the world began cultivating tilapia in the mid-1900s. Today, all commercially important species of Tilapia belong to the genus *Oreochromis*, and 90% are Nile tilapia, which are commercially produced outside of Africa. Some of the remaining species, such as blue tilapia (*O. aureus*), Mozambique tilapia (*O. Mossambicus*) and Zanzibar tilapia (*O. urolepis hornorum*), represent smaller shares of the commercial tilapia farming market. Worldwide production of Tilapia increased to 1,177,000 metric tons in 2016 and has become the second-highest production in terms of freshwater culture worldwide[11]. A limited amount of research has been conducted on marine Tilapia fish exposed to phenanthrene[12]. Hence, this study focused on the physiological alterations in marine cultured tilapia fish (*O. spilurus*) exposed to different concentrations of phenanthrene in Saudi Arabia.

2. Materials and Methods

2.1 Experimental Design and Fish Treatment

A total of one hundred and twenty mature Marine Tilapia (*Oreochromis spilurus*) were acquired from the Jeddah Fisheries Research Centre (JFRC), situated at coordinates 21°48'22.9"N, 39°02'07.7"E in the northern region of Jeddah. The fish were distributed at random into four groups: group 1 served as the control group, while groups 2, 3, and 4 were subjected to phenanthrene treatment. The total number of fish in each aquarium was 10, with 3 replicate for each treatment. Every cohort of fish was transferred to the laboratory and housed in glass aquariums measuring 70 cm x 40 cm x 51 cm, which were filled with 16.8 L of aerated

seawater. The water parameters, including temperature, pH, and salinity, were determined using the APHA techniques[13]. The measured values were 27±1°C for temperature, 7.1±0.5 for pH, and 41±0.5% for salinity. Before conducting studies, the fish were acclimatized to the laboratory environment for two weeks[14]. The four groups of fish, labeled A, B, C, and D, were subjected to varying concentrations of phenanthrene (0, 10, 25, and 50 mg/L-1) for a duration of 8 weeks.

2.2 Test Solutions and Food Preparations

Phenanthrene crystals (99.5% pure, Sigma) were first dissolved in absolute ethanol, then stock solution (1 mg mL^{-1}) was prepared and stored at -4°C in amber bottles. The stock solution was diluted with distilled water to prepare the test solutions of different concentrations. For the preparation of the phenanthrene-spiked fish food, the stock solution was diluted to the desired concentrations and mixed with fish food (TetraMin, Melle, Germany). The test dose concentrations were 0, 10, 25 and 50 mg/ L^{-1} for phenanthrene in treatments. These concentrations were set based on the US-EPA maximum permissible limit of the chemical in the environment. The mixed food was stored in Teflon-lined tubes to avoid phenanthrene becoming stuck to the tube wall. Then, the tubes were capped and kept on rollers at -4 °C for 24 hours to ensure the food was properly and thoroughly mixed with phenanthrene. The tubes were opened occasionally to remove the ethanol vapours. After 24 hours of mixing, the food was centrifuged to eliminate the water and then frozen[15]. The phenanthrene solutions and spiked food required careful handling due to their light-sensitive nature, necessitating storage in a dark environment[16].

2.3 Treatment of Fish with Phenanthrene Mixed Food

The spiked food was mixed with different concentrations of phenanthrene (0, 10, 25 and 50 mg L^{-1}) and fed to the fish twice a day for 8 weeks[15] (Ching-Yi et al., 2010).

2.4 Length and Weight of Fish

Fish weight (g) and length (cm), were measured using ordinary measuring scales described by Edwin et al. (2015).

2.5 Fish Oil Preparation

The fish oil of five fish from each treatment group was extracted in the Animal Physiology Laboratory, Department of Biological Science, King Abdulaziz University. The marine tilapia fish were beheaded, the tail was removed, and. The fish fillets were minced and placed in 40-ml centrifuge tubes with round bottoms. It was then centrifuged at 10,000 rpm for six hours until two upper layers (oil and aqueous) were observed. The oil was isolated by puncturing the bottom of the centrifuged tubes, then drained into a clean glass vial. Samples were preserved in nitrogen gas and stored in a deep freezer at -20 °C prior to further analysis[17] (Edwin et al., 2015).

2.6 Fluorescence Study

Fluorescent compounds were extracted by thawing, mixing and then vertexing the fish oil. Then, 50 µl of fish oil was mixed with 1.15 µl of ethanol in a 1.5 ml micro centrifuge tube. The mixture was then vortexed continuously for one minute, and the fish oil was separated by centrifuging for 20 minutes at 13,000 rpm. The fish oil accumulated in the upper-most layer in

the tube, and thereafter, 1 ml of the supernatant was isolated for further analysis. The fish oil samples were analysed for levels of phenanthrene compounds with a Perkin-Elmer LS55 spectrophotometer, with Hellma fluorescence (200-2,500) and quartz cuvettes (suprasil) having a 10 × 10 mm path length[17].

2.7. Liver Histology

The livers of the control and treated fish were fixed in 10% neutral-buffered formalin for 12-24 hours. The samples were then subjected to common histological assessment (desiccated and rooted in paraffin). Small 5 µm sections were excised and stained with haematoxylin and eosin[18,19].

2.8. DNA Extraction and Analysis

Genomic DNA from the treated and control fish livers were extracted and purified according to 'Qiagen Kit' protocol[20]. The DNA of fish livers were exposed to RAPD screening with four random oligonucleotide primers (Table 1). RAPD-PCR was performed on fish liver DNA samples (control, 10 mg L^{-1}, 25 mg L^{-1} and 50 mg/ L^{-1}) using four different primers according to the procedure described by Williams et al. [21].

Table 1. [DF1]**RAPD-PCR primers sequences used for amplification.**

Primer	*Codes*	*Sequence*	*CG%*
P1	B06	5`-TGCTCTGCCC-3`	60
P2	B07	5`-GGTGACGCAG-3`	70
P3	C19	5`-GTTGCCAGCC-3`	70
P4	B04	5`-GGACTGGAGT-3`	60

P = Primer

2.9. Statistical Analysis

ANOVA and descriptive statistical analysis were conducted using the statistical package Statistix version 8.1 (Tallahassee, Florida, USA).

3. Results

3.1. Length and Weight of Fish

The mean length and weight of the fish exposed to different treatments are presented in Table 2. The results exhibited a significant ($P < 0.05$) decrease in fish length associated with phenanthrene concentrations of 25 and 50 mg L^{-1}, while no significant changes were recorded at 10 mg L^{-1}. In addition, 10, 25 and 50 mg L^{-1} concentrations of phenanthrene significantly decreased the fish weight compared to the weight of the control fish, while no significant decrease was found between phenanthrene concentrations of 10 and 25 mg L^{-1}. The lowest mean length of 7.09 cm and the lowest weight of 6.53 g were found in fish treated with 50 mg L^{-1} of phenanthrene.

Table 2. Mean values of Morphological characteristics, Length (cm) and Weight (g) of Marine Tilapia fish at different concentrations of phenanthrene.

	Growth parameters	
Concentration (mg/ L^{-1})	Length (cm)	Weight (g)
0	8.64_{a}[DF2]	10.95^{a}[DF3]
10	8.22_{a}	9.20^{b}
25	7.54_{b}	8.10^{b}
50	7.09_{c}	6.53^{c}

Means within columns with different superscript are significantly different at LSD (p<0.05)

3.2. Fluorescence Spectrophotometry of Fish Oil

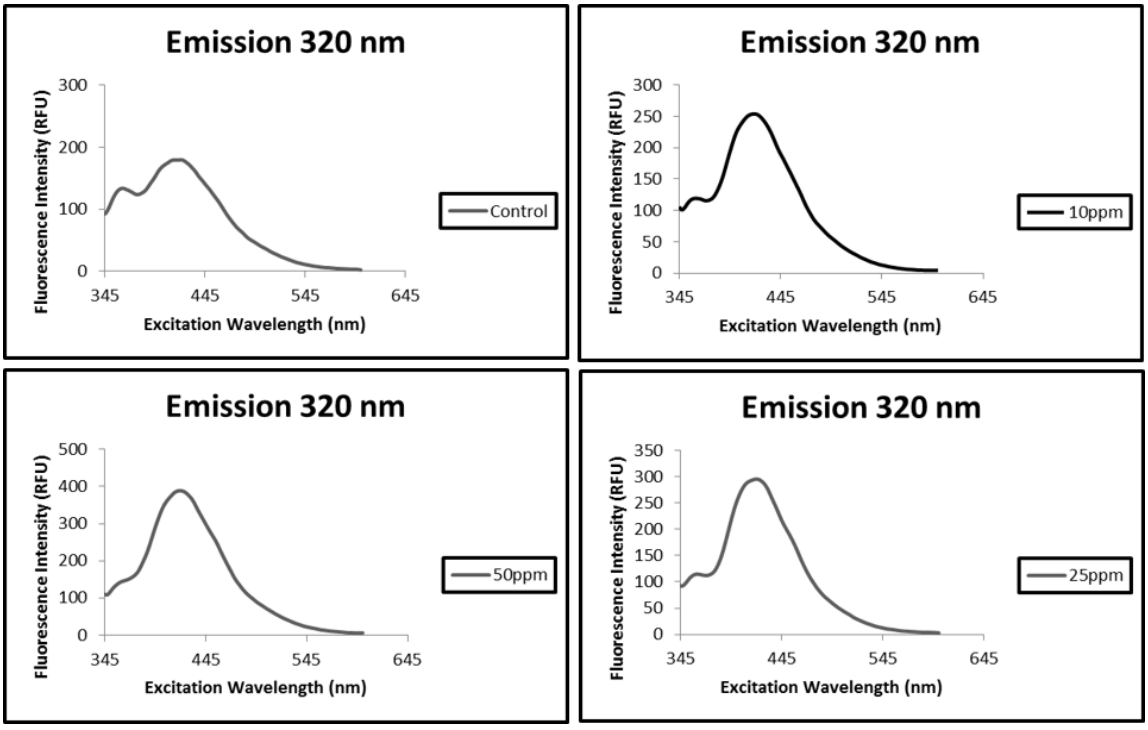

Fig. 2. Fluorescence spectrum at 320 nm emission of different phenanthrene doses.

The control sample was scanned for excitation from 200-600 nm. The descriptive statistic data are shown in Table 3, and the graphs are provided in Fig. 2. The control/oil fish (without phenanthrene) exhibited a maximum (peak) florescence intensity of 179.62 RFU at a wavelength of 421 nm and a mean SEM value of 80.96 ± 2.79. Fish treated with 10 mg L^{-1} of phenanthrene exhibited an asymptotic RFU of 253.87 at an emission wavelength of 418.5 nm, and its highest mean SEM value was 99.97 ± 3.69**.** Samples from fish treated with relatively high concentrations of phenanthrene level (25 mg L^{-1}) exhibited a higher peak RFU value of 295.57 at an emission wavelength of 419.5 nm and had a relatively higher mean SEM value of 109.66 ± 4.30.

Table 3. Showing the Mean ± SEM, Minimum and Maximum for different concentrations of phenanthrene.

Treatment (Phenanthrene mg L^{-1})	Mean ± SEM	Minimum	Maximum
0	80.96 ±2.79	2.13	179.62
10	99.97 ± 3.69	4.29	253.87
25	109.66 ± 4.30	2.94	295.57
50	150.11± 5.61	5.83	388.59

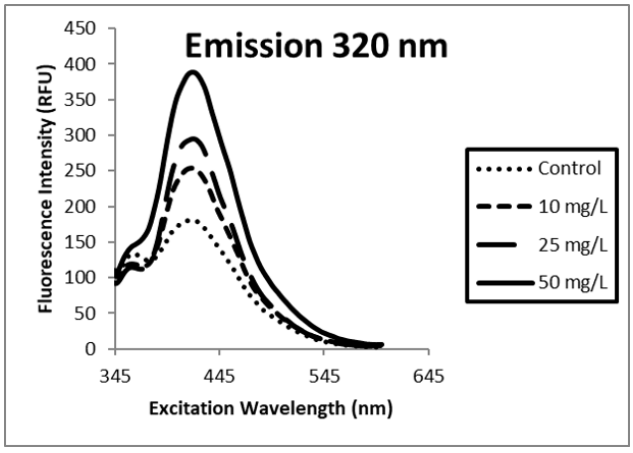

Fig. 3. Comparison of different concentrations of phenanthrene in fish oil.

Moreover, fish treated with the highest concentration of phenanthrene (50 mg L^{-1}) exhibited the highest RFU value of 388.59 at a wavelength of 419.5 nm and the highest mean SEM value of 150.11 ± 5.61. Comparatively, as the phenanthrene treatment dose increased, the concentration of phenanthrene found in the fish samples also increased (Fig. 3).

3.3. Liver Histology

The liver section from the control specimen was observed with a 40x light microscope, and digital snaps were captured. No alteration effect was observed in the liver cells of the control sample. Hepatocytes were normal in size and shape with cytoplasm and nuclei (Fig. 4A). The examination of the control fish liver confirmed the existence of hepatocytes with central round nuclei and polygonal shape, with sinusoids that occupied the gaps between the plates of hepatocytes.

After preparation of the slides with liver samples from fish treated with phenanthrene (10 mg L^{-1}), a slight change in shape and size of the cell and cellular organelles were observed with the light microscope (Fig. 4B). Other alterations like nuclear hypertrophy as well as the cell have also shown the same phenomenon to some extent. Relaxation of sinusoids was also observed (black circle) with blood infiltration. Similar observations were found in samples from fish treated with 25 mg L^{-1} of phenanthrene. The microscopic study revealed various changes, including nuclear hypertrophy, cell hypertrophy, nucleus degeneration (orange arrow in Fig. 4C) to some extent and intensity of dilation of sinusoids (black arrows in Fig. 4D).

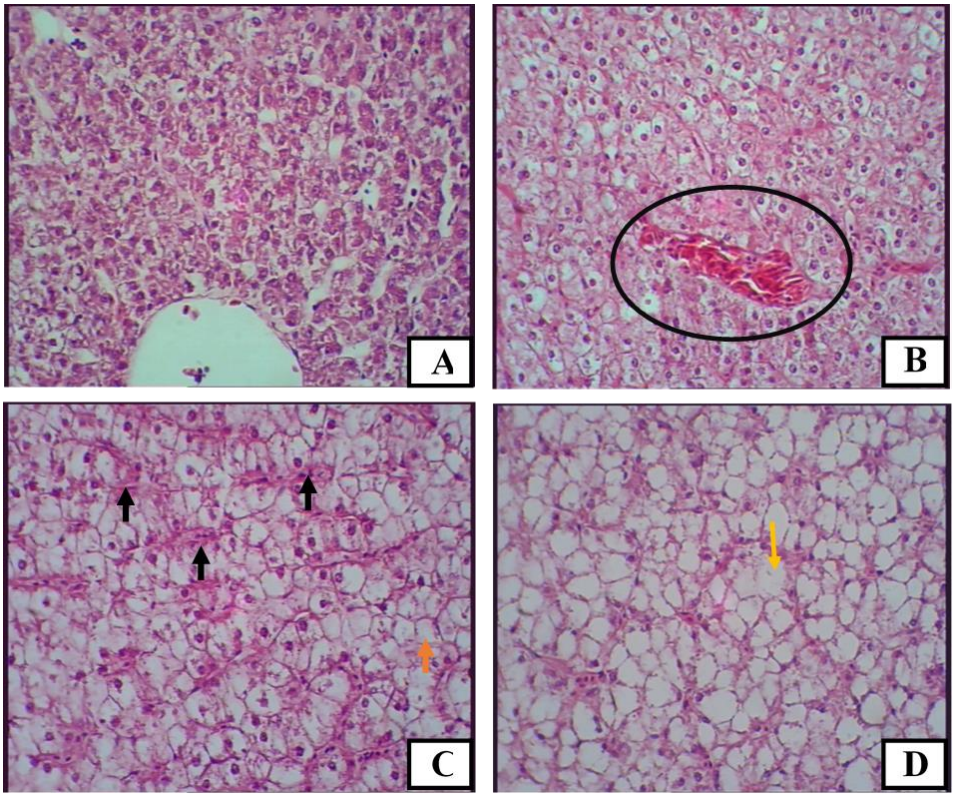

Fig. 3. Histological structure of liver (A) showing liver cells of control. (B) Relaxation of sinusoids and other cell changes in fish liver treated with 10 mg L-1 phenanthrene. (C) Showing alteration intensity in nuclear hypertrophy, cell hypertrophy, degeneration of the nucleus (Orange arrow) and intensity of dilation of sinusoids (Black arrows). (D) Cell and nucleus hypertrophy, expansion in sinusoid, cytoplasmic degeneration also vacuolation and melano macrophages aggregations (Yellow arrow).

Observations using the light microscope also revealed that the alteration in fish liver became more severe as phenanthrene doses increased. The change in cell shape, size and structure are clearly seen in Fig. 4D. Cell and nucleus hypertrophy, sinusoidal expansion, cytoplasmic degeneration and vacuolation, and melano-macrophage aggregations were observed. Ultimately, the impacts were the most severe in samples from the fish subjected to the highest concentration (50 mg L^{-1}).

3.4. Molecular (RAPD- PCR) Analysis

All four primers (P1, P2, P3 and P4) used in this study were successfully amplified with polymorphic bands for one control and three treated samples, as shown in Fig. 5. From our results, it seems that primer 1, 2 and 3 showed the same band profiles, but primer 4 exhibited some differences in the band profiles. RAPD using primer 4 had 3 bands in the control sample, 4 bands in the sample from group-2 fish treated with phenanthrene (10 mg L^{-1}), 6 bands in the sample from group-3 fish treated with phenanthrene (25 mg L^{-1}) and 9 bands in the sample from group-4 fish treated with phenanthrene (50 mg L^{-1}).

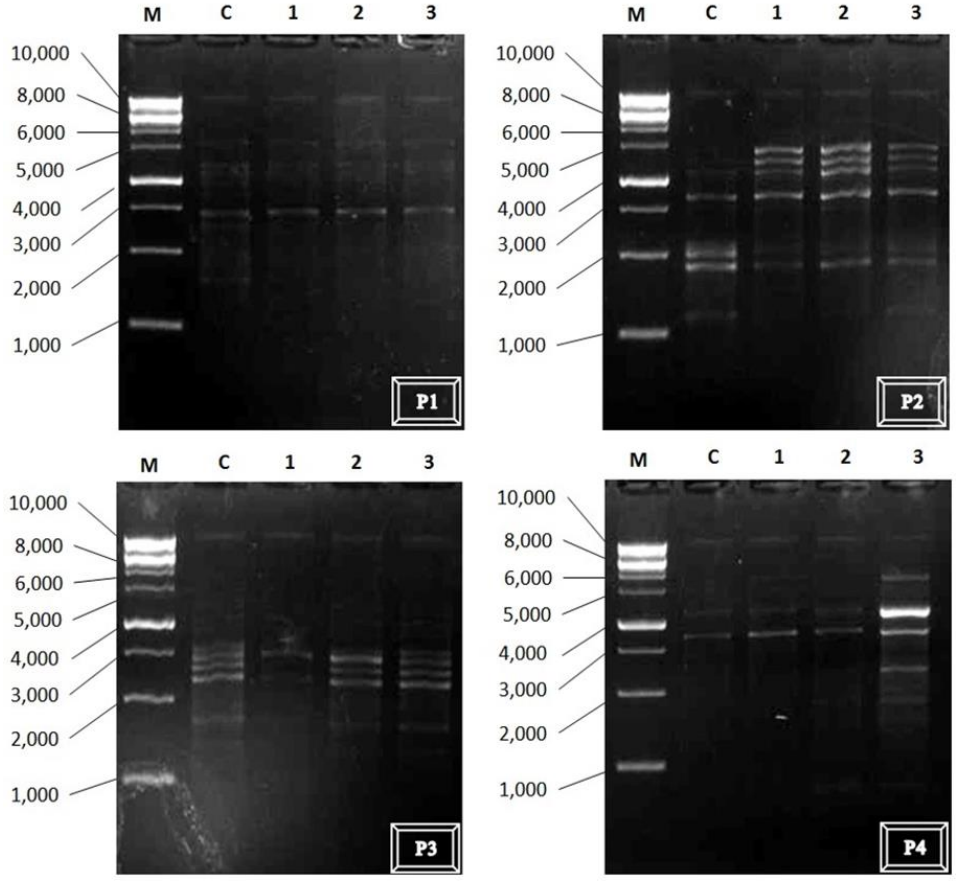

Fig. 5. Molecular liver DNA, PCR amplification by using RAPD assay.

4. Discussions

This study is primarily focused on the detection and evaluation of the effects associated with incremental increases in phenanthrene treatment concentrations on fish tissues and oil. For this research, marine tilapia (*O. spilurus*) was selected due to their wide range of adaptability in nature and higher survival rates in poor-quality water[10]. Conclusively, the results revealed significant effects ($P < 0.05$) of phenanthrene in fish tissues and oil, which confirmed possibility of accumulation of phenanthrene in the biological system of the treated fish.

The impact of phenanthrene hydrocarbon on the growth (length and weight) was found to be significant ($P < 0.05$). The data indicated that weight was more affected than length. The weight gain was significantly less in fish treated with phenanthrene than that of the control fish, which is consistent with Jung-Hoon et al. [22] who reported a significant decrease in weight gain of flounder fish exposed to different concentrations of phenanthrene. Another study by Chávez-Veintemilla, and Val[23], showed that Tambaqui fish (*Colossoma macropomum*) experienced decreased growth and mass when exposed to habitats contaminated with phenanthrene, even at low concentrations and for short durations. The authors illustrated growth, length, and mass reduction due to increased feed conversion following four weeks of waterborne phenanthrene exposure and decreased feed efficiency. Moreover, they hypothesized another factor to weight loss related to detoxifying mechanisms of fish exposed to phenanthrene that need energy

mobilization. They assumed that weight loss might occur if energy mobilization demands energy reserves, especially if food is insufficient to maintain organic equilibrium.

This may be associated with the possible negative effect of phenanthrene on fish health and major physiological functions. Due to its lipophilic nature, fish oil may accumulate and hold hydrophobic PAHs more efficiently than other biological components of the fish. This characteristic of fish oil allows the impact of oil spills to be tracked for longer periods of time. The use of fluorescence spectroscopy is effective due to easy sample preparation, efficient and rapid analysis and increased output compared to that of GC-MS and HPLC-F[24]. Prior to spectrophotometric analysis of fish oil, the emission constant was fixed at an excitation wavelength of 320 nm.

The results of our analyses also revealed a significant rise in the peak of florescence intensity in fish treated with incrementally increasing phenanthrene doses compared to fish in the control group due to the accumulation of phenanthrene in fish tissues. The increase in the RFU value between the control group and group 2 (10 mg L^{-1}) was relatively higher than the RFU increase between group 2 and group 3 (25 mg L^{-1}), and the highest increase was observed between group 3 and group 4 (50 mg L^{-1}). This might be due to the direct proportionality between the levels of phenanthrene and fluorescence intensity (RFU) of the absorbed spectrum. Our results are consistent with the findings of Edwin et al. [17], who studied menhaden fish oil. The oil of this fish was used in a biomonitoring technique that employed fluorescence spectroscopy to detect crude oil contaminants. They detected the PAH-like compounds in the oil by using spectramax with similar results. Similar results were also reported by Pathiratne and Hemachandra[12], who investigated the bile fluorescence arrangement in Nile tilapia by using fixed wavelength and synchronous fluorescence methods. Nile tilapias were used as an effective approach for biomonitoring of tropical water pollution. They confirmed that the PAH compounds are taken up in the fish body and released in bile, which was based on corresponding fluorescence data that were significantly higher in the PAH-treated samples compared to those of the control samples.

In this study, alteration or damage of the liver cells became more severe with increases in the concentration of phenanthrene. Hence, the highest intensity of phenanthrene was observed in the fish exposed to 50 mg L^{-1}. Our results were consistent with many previous findings related to the effects of different pollutants on fish liver[25,26,27]. Olojo et al. [28] pointed out the degeneration of hepatic cells and focal necrosis in the liver of *Clarias gariepinus* when treated with a heavy metal (lead). Similarly, when Oncorhynchus mykiss was treated with copper sulphate, the degeneration of liver cells was found to be triggered by the effect of copper sulphate along with the sinusoidal dilation and blockage in the blood veins[29].

The liver alteration may be due to the detoxification and excretion of pollutants that was responsible for some morphological changes in the liver[30]. Similarly, the vacuolization of the liver cells may be indicative of certain imbalances, such as the depletion of protein and glycogen, the compartmentalization of neutral lipids in the parenchyma cells and the rate of secretion into the circulatory system[31]. The compartmentalization of molecules boosts the size of the vacuole, inducing the pushing of the nucleus inside position, and this is normally correlated with nuclear atrophy[32]. The enlargement of liver sinusoids presumably showed the surge in blood volume for detoxification of the organisms in the liver[33]. The aggregation of

melanomacrophages in the liver cells of *O. spilurus* is a clear indication that the exposure of cells to phenanthrene triggered damage to cell structure and metabolic activities. Because of the increasing trend in the detoxification process, the hypertrophy in liver cells was due to the ultrastructure changes in the cell[34]. Moreover, deficient oxygen that results in gill erosion is the main cause of cellular degeneration in hepatic cells. Hemolysis and thrombosis of vascular dilation were found to be the result of the formation of stasis in blood vessels, which may also be responsible for the cellular degeneration and senescence of the liver cell[25].

Using RAPD markers may be an effective approach for many species from geographically and morphologically different populations in cases where little information about their genetics is available. The limitations related to pedigree data along with biological, morphological and marker-based estimation, was used to evaluate genetic variability, which had largely been developed by DNA markers, such as restriction fragment length polymorphisms (RFLP)[35] and random amplified polymorphic DNA[21]. The RAPD is an appropriate tool for DNA fingerprinting[36], although it suffered from a definite absence of reproducibility because of mismatch annealing[37]. The exploration of RAPD was found to be effective as a genetic marker, which improved the effectiveness of r-DNA techniques and utilized a single, arbitrary primer to amplify a number of DNA fragments to generate a discrete ‘‘fingerprint’’ resolved by gel electrophoresis[38]. In our study, RAPD-PCR was used because pollutants such as phenanthrene can damage the DNA of tilapia fish[39]. From our results, primers 1, 2 and 3 showed the same band profile, but primer 4 exhibited some differences in its band profile. RAPD using primer 4 had 3 bands in the control samples, 4 bands in the samples of group-2 fish treated with phenanthrene (10 mgL^{-1}), 6 bands in the samples of group-3 fish treated with phenanthrene (25 mgL-1) and 9 bands in the samples of group-3 fish treated with phenanthrene (50 mgL^{-1}). These results indicated that phenanthrene can affect the DNA of tilapia fish with a phenanthrene concentration of 50 mg L^{-1}.

5. Conclusion

Polycyclic aromatic hydrocarbons (PAHs) are a group of various chemicals with extensive and different groups of compounds including phenanthrene. We examined the effects of varying phenanthrene levels on marine tilapia to determine morphological, structural, functional, and genetic changes. Fish treated with the greatest concentration of phenanthrene had the lowest length and weight. Liver cells showed nuclear hypertrophy, cell hypertrophy, nuclei degradation, and sinusoidal dilation which intensified with rising phenanthrene concentrations. According to the fluorescence spectrophotometry examination of treated fish oil, a positive association was seen between the phenanthrene concentrations and the dosage of phenanthrene given to the fish. A RAPD-PCR experiment with four primers revealed probable genetic alterations in fish DNA that were exposed to high concentrations of phenanthrene. The results suggested that biochemical techniques might be employed as biomarkers to measure phenanthrene toxicity in fish and that tilapia could be a good marine environment monitoring organism.

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التغيرات الفسيولوجية والنسيجية المرضية التي يسببها الفينانثرين على سمكة البلطي البحرية *أوريوكروميس سبيلوروس* سعودي

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المستخلص. هدف هذه الدراسة هو التحقيق في تأثيرات تراكيز مختلفة من الفينانثرين (٠، ١٠، ٢٥، ٥٠ ملغ ل−١) المختلطة مع غذاء ملوث على أربع مجموعات علاجية من ٢٠ سمكة من تربية البلطي البحرية. تشمل هذه التأثيرات التغيرات الشكلية، الهيكلية، الوظيفية، والجينية. وُجدت تقليصات ملحوظة ($P<0.05$) في طول ووزن السمك (٧,٠٩ سم و٦,٥٣ غرام) في المجموعة المعالجة بأعلى جرعة من الفينانثرين (٥٠ ملغ ل−١). أشارت تحليل الطيف الضوئي للزيوت المعالجة من الأسماك إلى وجود ارتباط إيجابي بين تراكيز الفينانثرين والجرعة الممنوحة للأسماك. علاوة على ذلك، زادت التغيرات النسيجية في أنسجة الكبد مع زيادة تركيز الفينانثرين، بالإضافة إلى التغيرات الجينية. كشفت النتائج عن وجود الفينانثرين في النظام البيولوجي للأسماك، وتم تأكيد تأثيراته على أنسجة السمك. يُوصى بإجراء دراسات إضافية للتحقيق في سمية الفينانثرين على الأسماك وعلاماته الحيوية على المستوى البيوكيميائي.

الكلمات المفتاحية: سمك البلطي البحري، *أوريوكروميس سبيلوروس*، الهيدروكربونات العطرية متعددة الحلقات، الفينانثرين، الفلورسنت.