Middle-East Journal of Scientific Research 13 (10): 1406-1412, 2013
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2013.13.10.1282

Histological Responsesof Milkfish, *chanoschanos*, Liver Underpetroleum Hydrocarbonexposure

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Abstract: Oil pollution is a significant hazard for the marine environment. There is an increasing risk of amajor pollution spill in the Persian Gulf waters because of heavy transport of crude oil, petrol and its derivations. The aim of this study was to investigate the effect of exposure petroleum hydrocarbons on the milkfish liver at different concentration and different time points. After determination of lethal concentration of total petroleum hydrocarbons (Lc_{50}-96h), Collected specimens of Chanoschanos were exposed to different concentrations of petroleum hydrocarbons.The mainhistological alternation obsereved in this study include: sinusoid dilation, vacuolation,lipidiosis, melanomacrophage aggregation, necrosis, hepatocyte nuclear pleomorphism and bile stagnation.Our result showed that, The intensity of these histological changes wasinfluenced by theextent of the exposure period and concentration of pollution.

Key words: Petroleum Hydrocarbons · Pollution · Sublethal · LC_{50} · Histology

INTRODUCTION

In recent years, petroleum products pollution are one of the global environmental issue in aquatic ecosystems. The assessing and pridictionof the effects of petroleum pollution on aquatic environment are very urgent and important issue.

Petroleum hydrocarbonsand its products can enter the aquatic environment viadifferent sources such as petroleum extraction, transportation, urban runoff, biogenic hydrocarbons produced naturally, accidents involving and oil spills [1]. Petroleum hydrocarbons are lipophilic in nature andcan extremely uptake by awide spectrum of marine animals and accumulate in lipidic compartments like cellular membrane [2, 3], disturbe the physiological and physiochemical membrane propertiesand can integrated into biological system [4]. Many physiological processes such as enzyme function, muscle contraction and osmoregulation are directly dependent on the unique propertiesof biological membranes [2]. Some petroleum compoundesare potential to be biomagnified through the food chain [2]. Petroleum pollutants tend to accumulate more in the organism than the environment, therefor fish are largely being used for the assessment of the quality of aquatic environment and can be used as bioindicator to evaluate the environmental contamination levels [5-7].

Histological biomarkers were used for assessing the effect of petroleum hydrocarbons in aquatic organisms [8]. Histological study is a rapid method for detection of pollutants effects on various tissues of fish and it has been extensively used to determine the deleterious effects of hydrocarbons [8-10].

Nowadays, It is accepted that biomarkers are now useful tools in monitoring programs for the assessment of the impact of pollutants on marine organisms and ecological health [11-13]. Instead of measurement of accumulated pollutants in tissues, biomarkers can determine a more complete and more ecologically relevant informations about the potential impact of toxic pollutants on the living organisms and more important, allow the early detection of pollutants existence in ecosystems [14, 15].

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Exposure to pollutants may cause histological changes in fish organ, therefore histological investigations of exposed specimens may produce meaningful results [16]. The liver is a main detoxification organ and has two essential physiological role for both the basic metabolism and the accumulation, biotransformation and excretion of toxic substances in fish [17]. The harmful effect of pollutants on fish liver histology may depend upon the duration of the exposure (chronic or acute) and the concentration level of the specific pollutants as well as other factors such as temperature, age of fish, interaction with other pollutants, water chemistry and metabolic activity of the fish [18].

The toxicity of petroleum and derivation have been studied for a few species of fish [19, 20, 10] but no information is available about the toxicity of petroleum and derivatives with milkfish.

The first aim of present study was to determine the four days lethal concentration of petroleum hydrocarbons (PHCs) to *Chanoschanos* (Lc_{50}-96h).

Then this species exposed to sublethal concentration of petroleum hydrocarbons and the liver histological alteration was studied afterdifferent exposure time. This histological alteration can used as early biological markers for evaluation of polluted aquatic ecosystems.The effect of this pollution on the liver histological structure of this species have never been reported.

MATERIALS AND METHODS

Test Species and Acclimation Conditions: Milkfish, *Chanoschanos* (mean standard length:16.7±0.4 cm; mean weight: 64±1.2 g) were collected from Tiyab estuary and Shoor&Shirin river estuary (relatively unpolluted site) near the city of Bandarabbas (Iran). Fish were catched with cast nets and transferred to the experimental laboratory of the Persian Gulf and Oman SeaEcology Research Center. Specimens were washed with 0.1 % $kMNO_4$ solution to remove dermal infection, if any andwere acclimatized to laboratory condition for a period of 45 days before experimentation in well aerated concrete stock tank, containing natural filtered sea water.During the acclimatization process, the fish were fedtwice daily with commercial dry pellets (Havoor-Rash, Iran) containing 38% crude protein, 6% crude lipid, 13% ash and 10% moisture. The water was changed daily to discard the metabolic wastes. Fitness of the test animals is of prime importance in toxicity bioassay studies. Therefore diseased fish or fish showing any abnormal behavior were removed from the tank as soon as possible. Unnecessary handling of fish was alsostrictly avoided.

Determination of 96-H LC_{50} of Phcsand Selection of Exposure Concentrations: After acclimatization period, only active and healthymilkfish (mean standard length:17.82±0.6 cm; mean weight: 75.3±2.1 g) with no sign of distress and injury were used for this study. specimenswere randomly transferred from stock tank to polyethylene experimental tanks (280 l) which had continuous aeration. Four fish were randomly placed in each tank filled with seawater and allowed to acclimate to these conditions for two days. Fish were exposed to petroleum hydrocarbons (PHCs) concentrations of 2.4, 2.9, 3.4, 3.9, 4.4, 5.5 and 7ml/l plus one control treatment. The tested form of PHCswas the Gasoline which was obtained from a petroleum station in Bandarabbascity, Hormozganprovince, Iran. In Iran, the gasoline is the main fuels for marine vehicles. Three replications were carried out for each concentration (therefore 12 fishes per treatment). The tanks were randomly arranged in a laboratory.Experiment were run for 96h. The day before and during the experiment the fish were not fed. Throughout the experiment fish mortality was regularly monitored every 4h. Dead fish were immediately removed from the tank to prevent contamination of the test solution. To estimate 96-h LC_{50} value (LC_{50}: the concentration of the toxicant that caused 50% mortality in fish after a specific exposure time), the total mortality of milkfish in each concentration of the toxicant (each treatment) was recorded during 96h exposure. The data was analyzed by Finney's Probit Analysis Method Program to determined 96-h LC_{50} with ±95% confidence [21].

Experimental Design: In the present study, Young juvenile milkfishwere exposed to PHCs, at nonlethal concentrations of 0.26 ml/l (1/20 LC_{50}), 0.51ml/l (1/10 LC_{50}) and 1.02 ml/l (1/5 LC_{50}).The 30% of test water were renewed every other day and at the same time to remove the metabolic waste and fresh pollutant added to maintain the concentration of PHCs at a constant level. 21day exposures to this doses of PHCs were conducted and six samples wererandomly collected from each treatments onacute time (12, 24 and 96h) and subchronic time (7, 14 and21 days) at a same sampling time. The fishwere quickly captured and being anesthetized by immersion, until sedate, in a cloveessence solution. Standard length (L) and body weight (W) of each specimen were recorded.

Histology of the Liver: After each of the respective exposure periods, liver tissue was immediatelyremoved. Liver samples of each specimen at the same morphological region of each liver, to allow for comparative histological analysis, ($5\times5\times5mm^3$) were fixed in Bouin's solution for 24 h and they were transferred to 70% ethanol until routine histological processing for histopathological analysis using standard technique.The fixed tissues were dehydrated in ascending concentrations of ethanol series, cleared in xyleneandembedded in paraffin blocks. The blockes were sectioned with a rotary microtome (Leica RM2245, Germany) at 4.5 µm thickness. Sectioned tissues were stained with Hematoxylin and Eosin [22] and observed under light microscope(Olympus-CH_4O, Japan).A total of 10 sections were randomly observed from each treatment. Histopathological alteration was assessed using ascore ranging from - to ++++ depending on thedegree and extent of the alteration: (-) none, (+) mildoccurrence, (++) moderate occurrence, (+++) severe occurrence and (++++) very severe occurrence. Histopathologicalchanges induced by treatments in the tissues were photographed using Dino-lite photomicroscope.

RESULTS AND DISCUSSION

Water Analysis: The physical-chemical characteristics of the test seawater for all the experimental periods remained stable. The mean obtained values were:temperature, 23.4±0.6°C; pH, 8.1±0.2; dissolved oxygen concentration, 7.8±1 mg/l and salinity, 38.3±0.5p.s.u (practical salinity unit) [23].

LC_{50} Estimation: Figure 1 shows the relation between the PHCsconcentration and the mortality rate of *Cahnoschanos*. No mortality was observed during the 96h at PHC sconcentrations of 2.4, 2.9 and 3.4ml/l; mortality were 8.33, 33.33, 58.33 and 91.67% at 3.9, 4.4, 5.5 and 7ml/lrespectively.The results obtained from acute static toxicity experiments of PHCs upon milkfish were evaluated by using Finney's Probit Analysis and 96-h LC_{50} value ofPHCswith 95% confidence for milkfish was found to be 5.12ml/l.

Histopathology of the Liver: The liver histology of control specimens appeared relatively normal. The parenchyma itself is primarily composed of polyhedral hepatocytesthat, contain homogenous cytoplasm, typically with central nuclei and a prominent nucleolus.Venous blood enters the liver from the intestine via the hepatic portal veins and branches into capillaries known as sinusoids. Sinusoidsare lined with reticulo-endothelial cells which are in turn surrounded by hepatocytes.

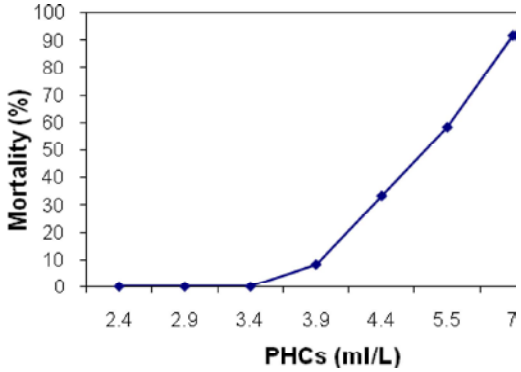

Fig. 1: The mortality percentage of milkfish (*Chanoschanos*) at different PHCs concentrations in 96h

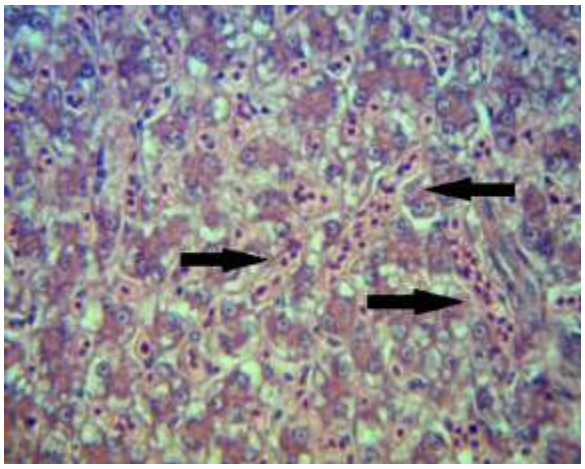

Fig. 2: Sinusoid dilatation(arrow),(2800X,H&E).

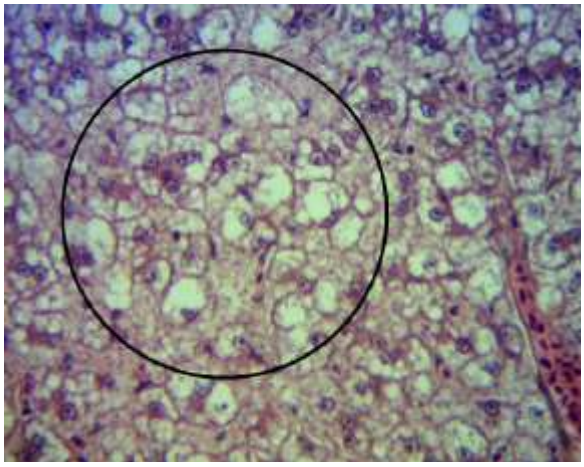

Fig. 3: Lipidiosis, liver section showing high level of lipid accumulation(circle),(2800X,H&E).

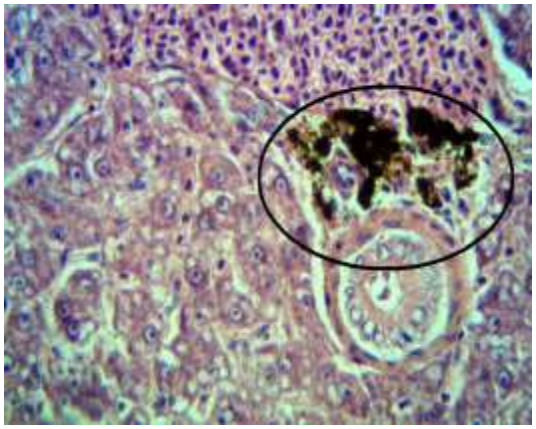

Fig. 4: Melanomacrophage aggregate (circle), (2800X,H&E).

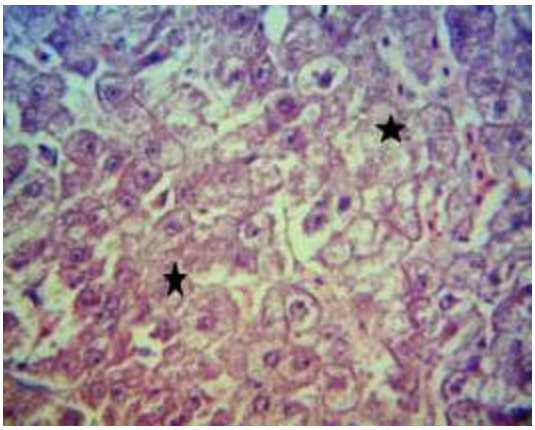

Fig. 5: Necrosis (*), (2800X,H&E).

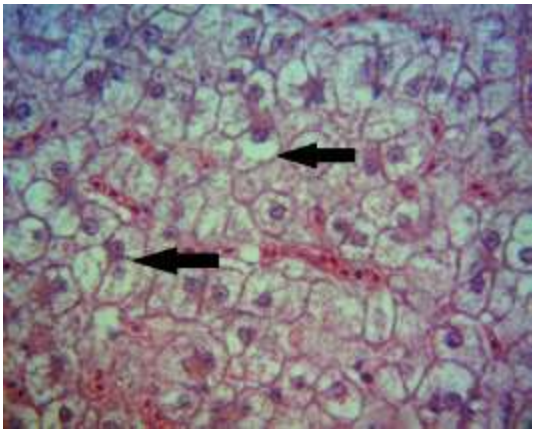

Fig. 6: Vacuolation (arrow), (2800X,H&E).

Compared to the control specimens, various histological changes were identified in the livers of juvenile milkfish exposed to the petroleum hydrocarbons. PHCs induced alterations in the histoarchitecture of the liver.

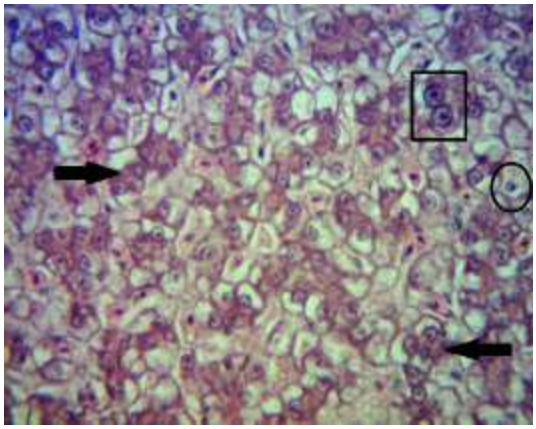

Fig. 7: Nucleuspleomorphism: Nuclear megalocity (□), pycnosis (○), Nuclear deformation (Arrow) (H&E).

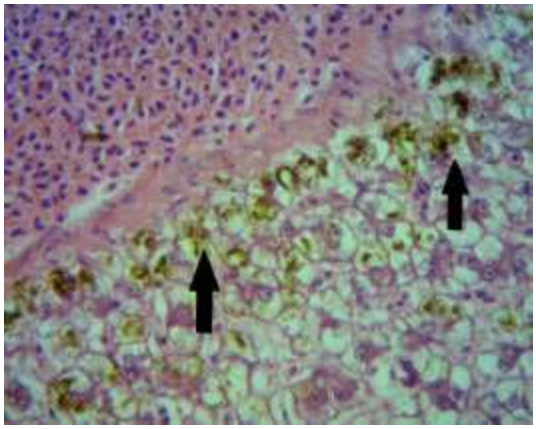

Fig. 8: Bile stagnation-brown granuls (arrow), (2800X,H&E).

The relevant histological alterations observed in the liver of milkfish were: Sinusoid dilation (Figure2), lipidiosis (Figure 3), Melanomacrophage aggregation (Figure 4), Necrosis (Figure 5), Vacuolation (Figure 6), Nucleus pleomorphism (Figure 7), Bile stagnation (Figure 8).The histological changes in the liver tissues of exposed fish at the different sampling time and concentration are summarized in Table 1-3.

According to results, liver of exposed milkfish showed varied histopathological alterations depending on the petroleum hydrocarbons concentration and duration of exposure.

The blood sinusoids are dilated between the cords of hepatocytes. Melanomacrophages are cells that belong to the unspecific immune system. The increase in MMCs has been related to a stress response which might result from exposure to toxic compounds [24].

Table 1: Semiquantitative scoring of liver lesion in *Chanoschanos* during acute and subchronic exposure of 1/20 LC_{50} PHCs

	Acute exposure (h)			Subchronic exposure		
Lesion (days)	12h	24h	96h	7d	14d	21d
Vacuolation	-	+	+	++	++	+++
Sinusoid dilatation	-	+	+	++	+++	+++
lipidiosis	-	-	+	++	++	+++
Nucleus pleomorphism	-	-	+	+	++	++
Bile stagnation	-	-	-	+	++	++
Melanomacrophage aggregation	-	-	+	+	++	++
Necrosis	-	-	-	+	+	++

Score value: None (-), mild (+), moderate (++), severe (+++), very severe (++++).

Table 2: Semiquantitative scoring of liver lesion in *Chanoschanos* during acute and subchronic exposure of 1/10 LC_{50} PHCs

	Acute exposure (h)			Subchronic exposure		
Lesion (days)	12h	24h	96h	7d	14d	21d
Vacuolation	-	+	++	++	+++	++++
Sinusoid dilatation	-	+	++	+++	+++	++++
lipidiosis	-	+	+	++	++++	++++
Nucleus pleomorphism	-	-	+	++	++	+++
Bile stagnation	-	-	+	++	++	+++
Melanomacrophage aggregation	-	-	+	++	++	+++
Necrosis	-	-	+	+	++	+++

Score value: None (-), mild (+), moderate (++), severe (+++), very severe (++++).

Table 3: Semiquantitative scoring of liver lesion in *Chanoschanos* during acute and subchronic exposure of 1/5 LC_{50} PHCs

	Acute exposure (h)			Subchronic exposure		
Lesion (days)	12h	24h	96h	7d	14d	21d
Vacuolation	-	+	++	+++	++++	++++
Sinusoid dilatation	-	+	++	+++	++++	++++
lipidiosis	-	+	++	+++	++++	++++
Nucleus pleomorphism	-	-	+	++	+++	++++
Bile stagnation	-	-	+	++	+++	++++
Melanomacrophage aggregation	-	+	++	+++	++++	++++
Necrosis	-	-	+	++	+++	+++

Score value: None (-), mild (+), moderate (++), severe (+++), very severe (++++).

According to [16,25] Vacuolation of hepatocytes are associated with the inhibition of protein synthesis, energy depletion, disaggregation of microtubules, or shifts in substrate utilization.Hepatocyte necrosis is a common pathologic response to toxin exposure in the liver. In this study, all treatments led to hepatocyte necrosis compared to control groups. Hepatocyte necrosis changes were observed in fish exposed over one days periods [26].Lipidiosis (the excessive accumulation of fat in the cytoplasm) is characteristic of many exposed livers. The large vacuole in the cell forces the nuclei to the periphery of the hepatocyte and this condition is usually accompanied by nuclear atrophy [8,16].

Nuclear pleomorphismischaracterised by marked nuclear enlargement, nuclear pynosis and deformation of spherical structure of nucleus in the present study. Hepatocellular pleomorphism are thought to be sublethally injured cells resulting from exposure to several types of toxicants [26].

Bile stagnation is characterized by the remains of the bile in the form of brownish-yellow granules in the cytoplasm of the hepatocytes [27], indicates that the bile is not being released from the liver. This accumulation of bile indicates possible damage to the hepatic metabolism [28].

As a conclusion, thefindings of the present histological investigations demonstrate adirect correlation between petroleum hydrocarbons (PHCs) nonlethal concentrations and exposure time withhisto pathologicaldisorders and lesions observed in milkfish liver tissue.Therefore, histopathology is a useful biomarker for environmentalcontamination, since it discriminated between control andtest groups. The liver showed to bethe organ affected by the type of stressors to which thefish were subjected. The *Chanochanos* was shown to be appropriate for environmental monitoringand*in situ* tests because of its hardly nature to survive under laboratory conditions.

ACKNOWLEDGMENT

The authors would like to gratefully acknowledge the staff of aquaculture lab at *Persian Gulf and Oman SeaEcology Research Center* for their assistance with this project. Thanks go to the staff at the department of marine biology at khorramshahr university of marine science and technology. Special thanks for *Dr. Movahedinia* for his guidance and for *AzadehAtabati* for histological help.

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