

Histopathological effects of water soluble-fraction of crude oil on liver tissue of fingerling beluga, *Huso huso* Linnaeus, 1754

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ABSTRACT

In this study the LC₅₀ 96h values of water-soluble fractions (WSF) of the Caspian Sea – exploited crude oil were determined in beluga, *Huso huso* fingerlings. The fingerlings averaged 2.42 ± 0.11 g in weight were exposed to WSF crude oil at concentrations 24.75, 27, 29.25, 31.5, 33.75, 36, 38.25 ppm. The treatments were performed in three replicates and all changes in the specimens were determined for each concentration. Water quality parameters of the seawater were tested and recorded. The LC₅₀ 96h value was found to be 34.87 ppm. In the other step, the fingerling beluga was exposed to three sub-lethal concentrations (13.93, 20.92, 27.90 ppm) of WSF of the Caspian crude oil representing 40, 60 and 80% of LC₅₀ 96h respectively, and control without crude oil. All treatments and control were conducted in triplicate. Three specimens of each treatment were sampled for histological studies every day. Results revealed that the fish liver tissue of all the treatments showed histological changes, in comparison to the control after 2 days. Even if the changes in the treatment exposed to 27.90 ppm (80% LC₅₀ 96h) WSF occurred in 24h after exposure. Histopathological findings in liver included cloudy swelling, pyknotic nuclei, Karyorrhectic nuclei, fatty vacuolation, fatty degeneration, hypertrophy of hepatocyte, necrosis, hypertrophy of bile ducts, cholestasis and bile stagnation, inflammation, fibrosis and cirrosis. These alterations were more conspicuous with increasing the soluble fraction of crude oil concentrations and exposing time. The results are of importance since much attention has been paid to the oil slicks, particulate or sedimentary pollutions, instead of potential toxic effects of water-soluble fractions of oil, which are more available to marine biota.

Keywords: *Beluga, Huso huso, WSF of crude oil, Caspian Sea, Histology*

INTRODUCTION

Petroleum oil provides ingredients for thousands of production that we use every day, but this involves various potential problems. In recent years, oil pollution has become a global environmental issue in oceanic and marine ecosystems and also inland aquatic breeding ecosystems which threatened greatly. The evaluation and prediction of the effects of oil pollution on water environment have become a very urgent and important issue (Engelhardt,

1983; Khan *et al.*, 1991). Pollution by crude oil is wide spread and a common problem and particularly endemic in countries whose economies are dependent on oil industry. Such pollution arises either accidentally or operationally wherever oil produced, transported, stored, processed or used. The constituents of crude oil are complex. It contains aliphatic, alicyclic, polyaromatic hydrocarbons, oxygen, nitrogen and sulphur containing substances. The other components include

toxic phenols and anilines (Traven, 1992). Sources of petroleum input to the sea are various and they can be categorized into four major groups, natural seeps, petroleum extraction, transportation and consumption. Accidents involving petroleum hydrocarbons spills occur frequently around the world and the annual worldwide estimative of petroleum input to the sea exceed 1,300,000 metric tons (NAP, 2003). The deleterious effects of petroleum are the result of physical fouling and the intake of water-soluble and insoluble hydrocarbons by aquatic biota (NAP, 2003). Only a relatively small fraction dissolves and becomes bio available. The water-soluble fraction (WSF) of crude oil and their derivatives products contains a mixture of polycyclic aromatic hydrocarbons (PAH), mono aromatic hydrocarbons often referred to as BTEX (benzene, toluene, ethyl benzene and xylenes), phenols and heterocyclic compounds, containing nitrogen and sulfur (Saeed and Al-Mutairi, 1999), and also heavy metals. Some petroleum-derived hydrocarbons are toxic to a wide spectrum of marine animals because they preferentially accumulate in lipid compartments like cellular membrane (Di Toro *et al.*, 2001), disturbing the physicochemical and physiological membrane properties (Sikkema *et al.*, 1994). All crude oils and many refined products in sufficient concentrations are poisonous to aquatic organisms. Direct killing of aquatic organisms can occur through coating with oil and by asphyxiation (Hoong *et al.*, 2001). In addition, the incorporation of finely dispersed particles of oil and oil products into organisms can negatively affect the body organs and systems, either directly or as a consequence of bioaccumulation processes (Lockhart *et al.*, 1996). The accumulation of soluble petroleum hydrocarbons in fish is extremely rapid (Gravato and Santos, 2002). Fish can be used as bio indicators to evaluate the environmental contamination levels of hydrocarbons, because these pollutants tend to accumulate more in

organisms than in the environment (Anyakora *et al.*, 2005). The early life stages of fish are particularly sensitive to xenobiotics. Acute toxicity tests with early life stages of fish are often used to determine legally applicable measurements of pollutants and to estimate their effects on aquatic biota (Westernhagen, 1989). Biochemical, physiological and histological biomarker, among others, have been used to determine the effects of petroleum-derived hydrocarbons in aquatic biota (Stephens *et al.*, 1997; Simonato *et al.*, 2008). Histology is a rapid method to detect effects of different pollutants in various tissues and organs of fish (Bernet *et al.*, 1999), and it has been extensively used to determine the deleterious effects of hydrocarbons (Brand *et al.*, 2001; Akaishi *et al.*, 2004; Simonato *et al.*, 2008). The organ most associated with the detoxification and biotransformation process is the liver, and due to its function, position and blood supply (Van der Oost *et al.*, 2003) it is also one of the organs most affected by contaminants in the water (Rodrigues & Fanta, 1998).

In the present study, beluga fingerlings, one of the most commercially important fish species in the Caspian Sea, was used as an experimental fish. The aim of this study was to determine the acute lethal concentration (LC₅₀96h) of the water soluble fraction (WSF) of the Caspian crude oil to investigate the damage caused by toxic sub-lethal concentrations of WSF, and to describe histological changes in liver tissue during the experimental period.

MATERIALS AND METHODS

Preparing Fish

A total of 600 individual of beluga fingerlings prepared from Shahid Rajaie Sturgeon Hatchery Center, Mazandaran Province, Iran (initial weight \pm SD= 2.42 \pm 0.11g) were used in this study. Fish were held in 30 L tanks containing 10 fish per tank with semi-static water conditions including mean temperature 24 \pm 1.2 °C, dissolved oxygen 8.6 \pm 0.25 mg L⁻¹ and pH 8.4 \pm 0.11. Other water quality parameters

including ammonia, nitrite and carbon dioxide were <0.1 , <0.1 and <5 mg L⁻¹, respectively. Fish were acclimated to experimental conditions for a few weeks prior to the experiments.

Preparation of Water-Soluble Fraction (WSF)

The water soluble fraction (WSF) of Caspian Sea crude oil (API=34.5, SP.Gr=0.850) was prepared as described by Anderson *et al.* (1974). Briefly, 1 part of crude oil was added to 10 part sea water in a glass container and stirred gently for 20 h at room temperature), adjusting the vortex of the mixture to no more than one-third of the height of the mixture from the oil-water interface. The mixture was then allowed to settle for 1 h to separate water and upper oil phases with some modifications. After 1 h resting, the WSF was ready to be used in the toxicity tests. The flask remained tightly capped during the entire process to minimize evaporation. The main and experimental concentrations of WSF were measured by Gas Chromatographer (GC×GC).

Acute Toxicity Tests

Acute toxicity tests were carried out beluga fingerlings in a 96h period. Photoperiod regime was 12 h L/12 h D. Temperature and salinity were kept at 24 ± 1.2 °C and 9 ppt during the tests (Binaii *et al.*, 2013). Throughout the experiment, oxygen and pH were measured daily. Aeration was withheld during the toxicity tests. Preliminary tests were performed in order to define suitable lethal concentrations of the toxicant. Concentrations of WSF for treatments were 24.75, 27, 29.25, 31.5, 33.75, 36, 38.25 ppm plus a control without crude oil. All treatments and controls were conducted in triplicate, where 30 fish were randomly distributed for each treatment (n=10). Fish mortality was monitored every day. The collected data from the experiments were used to estimate the 96 h LC₅₀ of (WSF) crude oil. To calculate the LC values, the mortality in each treatment was determined and analyzed by probit value

analysis method. The LC₅₀ 96h was calculated as 34.87ppm.

Exposure to Water Soluble -Fraction of Crude oil

Fish were divided into four treatments in three replicates. One hour before the fish transfer, different concentrations of WSF were added to the experimental glass aquaria as below:

T1: Served as a control.

T2: Exposed to 13.93 ppm (40% LC₅₀96 h)

T3: Exposed to 20.92 ppm (60% LC₅₀96 h)

T4: Exposed to 27.89 ppm (80% LC₅₀96 h)

All treatments and controls were conducted in triplicate.

Histological Analysis

Each 24h, three specimens of each experimental group were sampled for histological studies.

Liver was necropsied and fixed in 10% neutral buffered formalin, dehydrated in graded series of ethanol, cleared in xylene and embedded in paraffin. Five micron sections were cut and stained with hematoxylin and eosin (H&E) for histopathological examination. Slides were examined by light microscopy (Olympus BH-2 microscope) and the images were obtained by a digital camera (Bagherzadeh Lakani *et al.*, 2013).

Statistical Analysis

The acute toxic concentrations of water soluble fraction of crude oil in *Huso huso* was determined by probit analysis in SPSS software (Ver.18)

RESULTS

The histopathological examination of *H. huso* exposed to water soluble fraction of crude oil revealed some alterations in liver tissue. These alterations were more conspicuous with increasing the water soluble fraction of crude oil concentrations and also the exposure times:

T1: The control liver tissue had normal structure (Fig.1.A).

T2: Fish exposed to 40% LC₅₀96h of water soluble fraction of Caspian crude oil for 24h showed normal structure and after

48h, the increase of lococyte and cloudy swelling was occurred. Liver tissue of this treatment after 72h showed pyknotic nuclei and necrosis and after 96h, focal necrosis of hepatic tissue was observed (Fig.1.B-E).

T3: Liver tissue of *H. huso* exposed to 60% LC₅₀96h of water soluble fraction of crude oil for 24h showed normal structure and after 48h showed Fatty degeneration, Hypertrophy of hepatocyte and necrosis. After 72h, Coagulation necrosis, inflammation and fibrosis were observed and after 96h hypertrophy of bile duct,

inflammation fibrosis and cirrhosis occurred (Fig.2.A-D).

T4: In highest concentration of water soluble fraction of crude oil (80% LC₅₀96h) after 24h, macro fatty vacuolar and cloudy swellings were observed in liver tissue. After 48h necrosis and karyorrhexis in nuclei occurred. After 72h, necrosis and hyperplasia of bile duct, cholestasis and bile stagnation were observed and after 96h high necrosis and cirrhosis occurred (Fig.3.A-D).

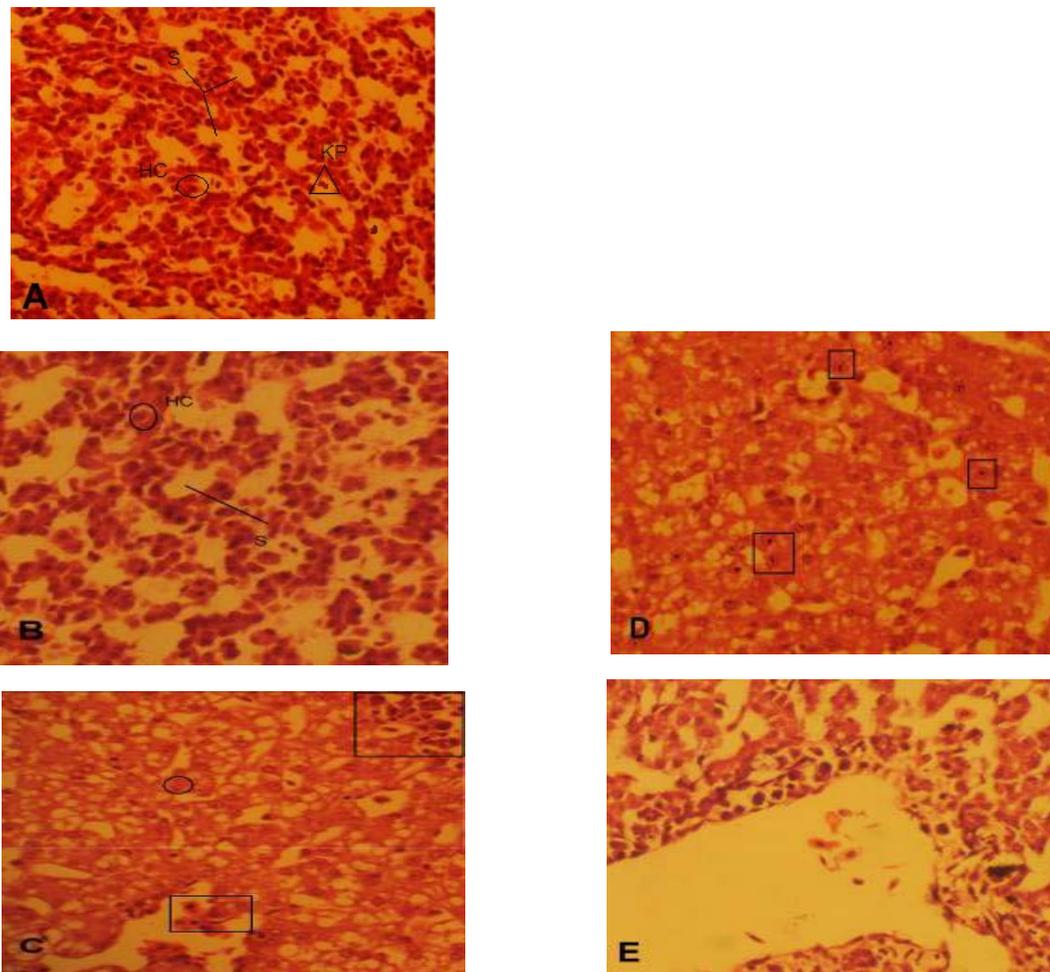


Fig.1. Photomicrographs of the liver of *H. huso* (A) control treatment showing normal structure in liver tissue (S: sinusoid, HC: hepatocyte, KP: kupfer); (B) exposed to 40%LC₅₀ 96h of water soluble fraction of crude oil for 24h, showing normal structure (S: sinusoid, HC: hepatocyte) ; (C) exposed to 40%LC₅₀96h of water soluble fraction of crude oil for 48h, showing increased lococyte (square) and cloudy swelling (circle) ; (D) exposed to 40%LC₅₀96h of water soluble fraction of crude oil for 72h, Showing pyknotic nuclei (square) ; (E) exposed to 40%LC₅₀96h of water soluble fraction of crude of crude oil for 96h, showing focal necrosis of hepatic tissue ; (H & E stain, 40x).

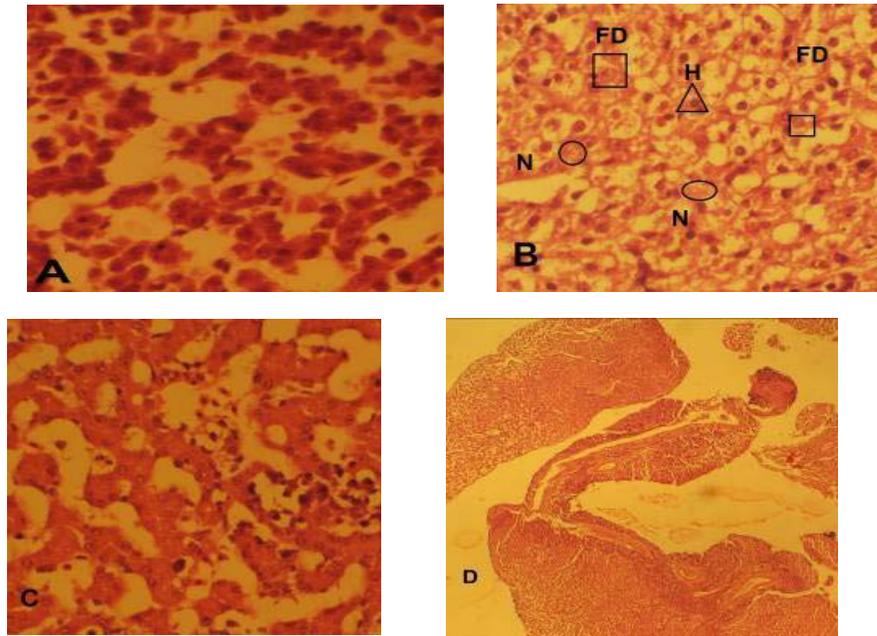


Fig.2. Photomicrographs of the liver of *H. huso* (A) exposed to 60%LC5096h of water soluble fraction of crude oil for 24h, showing normal structure ; (B) exposed to 60%LC5096h of water soluble fraction of crude oil for 48h, showing Fatty degeneration (FD), Hypertrophy of hepatocyte (H), necrosis(n) ; (C) exposed to 60%LC5096h of water soluble fraction of crude oil for 72h, showing Coagulation necrosis, inflammation and fibrosis ; (H and E stain, 40x) ; (D) exposed to 60%LC5096h of water soluble fraction of crude oil for 96h, Showing Hypertrophy of bile duct, inflammation and fibrosis, cirrhosis ; (H & E stain, 10x).

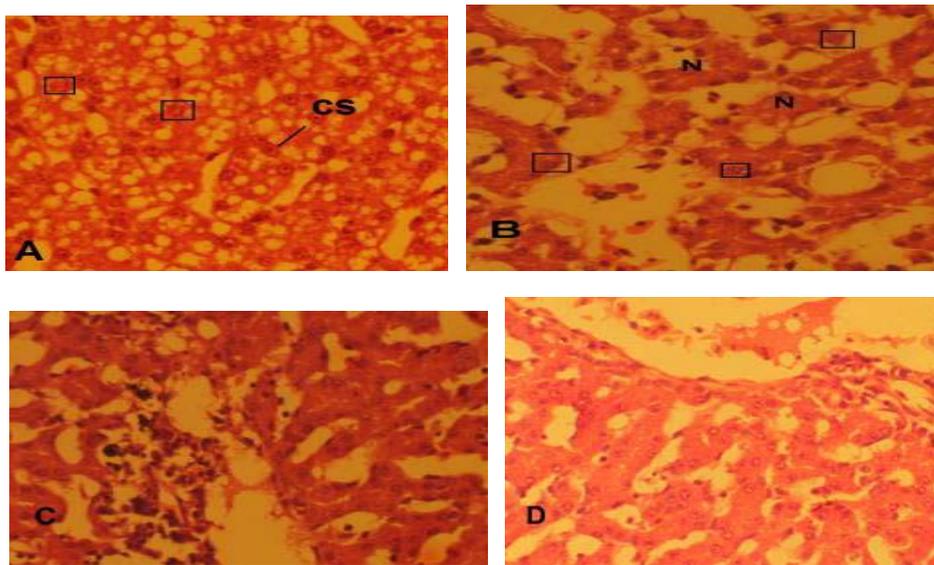


Fig.3. Photomicrographs of the liver of *H. huso* (A) fish exposed to 80%LC5096h of water soluble fraction of crude oil for 24h, showing macro fatty vacuolar(square), cloudy swelling(CS) ; (B) exposed to 80%LC₅₀96h of water soluble fraction of crude oil for 48h, showing necrosis(N), Karyorrhectic nuclei(square) ; (C) exposed to 80%LC₅₀96h of water soluble fraction of crude oil for 72h, showing necrosis, hyperplasia of bile duct, cholestasis and bile stagnation ; (D) exposed to 80%LC₅₀96h of water soluble fraction of crude oil for 96h, Showing highly necrosis and cirrhosis ; (H and E stain, 40x).

DISCUSSION

Release of petroleum and refined fuels into the environment have negative impacts, not only as a result of physical effects, but also as a consequence of toxicity of water-soluble fraction to biota. Even when exposure levels are not high enough to cause lethality, organisms may be affected by toxic available dissolved hydrocarbons (Rodrigues et al., 1998). Histopathological alterations can be used as indicators of the effects of various pollutants on the organisms including fish, and reflection of the overall health of the entire pollution. According to studies by Mohamed (2009) the exposure of fish to pollutants were resulted in several pathological changes in different tissues of fish. Pathological changes in organs and tissues are generally characterized by an obvious increase morphological and physiological, or functional, variability, both within a single organism and from one individual to another. The liver is the main organ of biotransformation and excretion of xenobiotics, and their presence rapidly results in structural, biochemical and molecular alterations (Bernet et al., 1999). The majority of the pollutions are biotransformed in metabolites by the liver, through enzymes from the soluble fractions of mitochondria and microsomes, and in some cases the metabolites are more toxic than the original product (Fanta et al., 2003).

The present study examined the histopathological changes due to three concentrations of Water soluble fraction of crude oil lower than LC₅₀ 96h and 1 control for 96 hour. Results revealed that the fish liver tissue of all the treatments showed histological changes, compared to the control after 2 days. The changes in treatments exposed to 27.90 ppm (80% LC₅₀96h) WSF occurred even in 24h after exposure.

According to the results of this study, pathological findings in liver included cloudy swelling, pyknotic nuclei, karyorrhectic nuclei, fatty vacuolar, Fatty degeneration, hypertrophy of hepatocyte,

necrosis, hypertrophy of bile duct, cholestasis, bile stagnation, inflammation, fibrosis and cirrosis. These alterations were more conspicuous by increasing the concentration of soluble fraction of crude oil and over time.

Alterations such as irregular shaped hepatocytes, cytoplasmic vacuolation and nucleus in a lateral position, close to the cell membrane, were also described in the siluriform *Corydoras paleatus* contaminated by organophosphate pesticides (Fanta et al., 2003). Vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver. Pacheco & Santos (2001) described increased vacuolisation of the hepatocytes as a signal of degenerative process that suggests metabolic damage, possibly related to exposure to contaminated water (Chang et al., 1998). The liver parenchyma of the animals exposed to the diesel oil showed signs of degeneration (cytoplasmic and nuclear degeneration, and nuclear vacuolation) besides the focal necrosis that was found in one fish confined (Simonata et al., 2008). These alterations are more severe and have been associated with the exposure of the fishes to contamination by metals, such as copper (Paris-Palacios et al., 2000) and mercury (Oliveira Ribeiro et al., 2002), and by polychlorinated biphenyls (PCBs), (Chang et al., 1998).

The present study shows that the histopathological changes in the liver result in metabolic problems. Evidence for this is the bile stagnation in the liver of some studied fish. This lesion, characterized by the remains of the bile in the form of brownish-yellow granules in the cytoplasm of the hepatocytes (Pacheco & Santos, 2001), indicates that the bile is not being released from the liver. This accumulation of bile indicates possible damage to the hepatic metabolism (Fanta et al., 2003). In this study an increase in the density of the melanomacrophage aggregates were found. This may generally be related to important hepatic lesions such as degenerative and necrotic processes

(Pacheco & Santos, 2001). Same results were observed in *Pleuronectes americanus*, exposed to PAHs (polycyclic aromatic hydrocarbons) and pesticides in urban areas on the USA coast (Chang *et al.*, 1998). The function of the melanomacrophages and lymphocyte in the liver of fishes remains uncertain, but some studies have suggested that it is related to destruction, detoxification or recycling of endogenous and exogenous compounds (Haaparanta *et al.*, 1996). The presence of bile stagnation and melanomacrophages in great quantity in the livers of *Prochilus lineatus* is strong evidence that these organs suffered structural and metabolic damage due to exposure to the diesel oil (Simonata *et al.*, 2008).

In the present study multiple necroses occurred in many of the samples exposed to WSF. Similar results reported by Spies *et al.* (1996), where the multiple necroses and variable number of lymphocytes in the liver of organisms exposed to a natural petroleum in Santa Barbara were observed. Histopathological abnormalities in the liver of fresh water and saltwater species exposed to petroleum hydrocarbons were also described in the literature (Akaishi *et al.*, 2004; Khan, 2003). According to these authors, the most important damage caused by hydrocarbons was necrosis with cellular inflammatory response in the liver. In this study in all treatments after 72h exposure necrosis of hepatic tissue occurred.

Marchand *et al.* (2008) studied on Histopathological alterations in the liver of the sharptooth catfish *Clarias gariepinus* from polluted aquatic systems in South Africa, Assessment of the liver tissue revealed marked histopathological alterations including: structural alterations (hepatic cord disarray), plasma alterations (granular degen, erosion and fatty degeneration) of hepatocytes; an increase in melanomacrophage centers; hepatocyte nuclear alterations; and necrosis of liver tissue, similar to results of the present study.

Many pollutants exist in the aquatic environment, for short or long periods, at sub-lethal levels. These levels are not noticed because they do not cause immediate fish mortality. However, the consequences of such effects are morphological and physiological, causing illness and reducing fitness for life (Fanta *et al.*, 2003). Therefore, the simple fact that a sub-lethal concentration is considered safe because it does not kill any fish, does not mean that it can be used indiscriminately, because, as we have seen in *H. huso*, the fishes showed illness. Results of the present study show that even sub-lethal concentration of water soluble fraction of Caspian Sea - exploited crude oil can cause histological damage in the liver of *H. huso* fingerlings. So while the LC₅₀96h of this pollutant determined as 34.87 ppm, the lowest experimental concentration (40% LC₅₀96 h = 13.93 ppm) can cause serious health risks in fingerlings of this commercially important species.

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تأثیر فاز محلول نفت خام بر شاخص‌های بافت شناسی کبد بچه فیل ماهیان (*Huso huso* Linnaeus, 1754) انگشت قد

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چکیده:

در این تحقیق میزان LC₅₀96h فاز محلول نفت خام دریای خزر برای بچه فیل ماهیان (*Huso huso*) انگشت‌قد با میانگین وزن $2/42 \pm 0/11$ تعیین شد. بچه فیل ماهیان انگشت‌قد در معرض غلظت‌های 36، 33/75، 31/5، 29/25، 27، 24/75، 20/92، 13/93 و 27/90 ppm فاز محلول نفت خام قرار داده شدند. تیمارها با سه تکرار در نظر گرفته شدند و تمام تغییرات نمونه‌ها در هر یک از غلظت‌های آلاینده تعیین شدند. شاخص‌های کیفی آب دریای مورد استفاده در آزمون ارزیابی شده و ثابت نگهداشته شدند. میزان LC₅₀96h معادل ppm 34/87 اندازه‌گیری شد. در مرحله بعد بچه فیل ماهیان انگشت‌قد در معرض سه غلظت زیر حد کشندگی (13/93، 20/92 و 27/90 ppm) فاز محلول نفت خام دریای خزر معادل 40، 60 و 80٪ LC₅₀96h قرار داده شدند و یک تیمار بدون آلاینده بعنوان شاهد در نظر گرفته شد. کلیه تیمارها و شاهد دارای سه تکرار بودند. هر 24 ساعت سه نمونه از هر یک از گروه‌های آزمایشی جهت مطالعات بافت‌شناسی تهیه شد. نتایج نشان داد که در بافت کبد ماهیان کلیه تیمارها در قیاس با شاهد پس از 2 روز تغییرات بافتی بروز نمود. اگرچه تغییرات در تیماری که در معرض 27/90 ppm (80٪ LC₅₀96h) فاز محلول نفت خام بود، پس از 24 ساعت بروز نمود. نتایج تغییرات بافتی کبد شامل تورم ابری، فشردگی هسته، تکه‌تکه شدن هسته، واکوئل شدن چربی، دژنراسیون چربی، هایپرتروفی هیپاتوسیت، نکروز، هایپرتروفی مجاری صفراوی، کلونستازیس و عدم حرکت صفرا، آماس، فیبروز و سیروز بود. این عوارض با افزایش غلظت فاز محلول نفت خام و زمان مواجهه با آلاینده افزایش نشان داد. اهمیت این نتایج از آنجایی است که بیشتر توجهات بجای اثرات سمی هیدروکربن‌های آروماتیک محلول که بیشتر در دسترس موجودات دریایی هستند، معطوف به لکه‌ها و ذرات نفتی و آلودگی رسوبات می‌باشد.

* مولف مسئول