



Research Article

A SCITECHNOL JOURNAL

Hepatic Parameters of Marine Fish *Rachycentron canadum* (Linnaeus, 1766) Exposed to Sublethal Concentrations of Water-Soluble Fraction of Petroleum

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Abstract

The aim of this paper was to evaluate the liver histological changes, hepatosomatic index and condition factor, as well as the activity of the enzymes alanine aminotransferase and aspartate aminotransferase after sublethal exposure 0.3 ppm of water-soluble fraction (WSF) of petroleum the period from 7 and 14 days. Observed an increase of hepatosomatic index after 7 and 14 days and reduced condition factor at 14 days of exposure, increased AST activity in serum at 14 days of exposure and liver histological alterations in 7 and 14 days, such as hypertrophy of nucleus of the hepatocytes, increase of density of the hepatic blood vessels, disarrangement of hepatic cords, cell atypia contour, intense cytoplasmic and nuclear vacuolization and hyperemia. We concluded that the evaluation of hepatosomatic index, histopathological alterations and activity of enzymes aspartate aminotransferase (AST) are sensitive to exposure to WSF, being good biomarkers. It is concluded, also, that the sub-lethal exposure to 0.3ppm WSF change of hepatic parameters of the fish species *Rachycentron canadum*, affecting the functioning of the biotransformation system. Knowledge of the changes and their adaptations will serve as the basis for the conservation of the species and more effective environmental monitoring.

Keywords

Fish; AST; ALT; Histological changes; Hepatosomatic index

Introduction

Petroleum is a natural product derived from the decomposition of organic matter promoted by chemical, geological and geochemical processes. Among the petroleum components, stands out polycyclic aromatic hydrocarbons that are water soluble molecules, highly volatile and toxic to fish [1] and the long chain hydrocarbons, which despite being less volatile and less toxic to fish, are more persistent in aquatic environments [2,3].

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Received: May 09, 2016 Accepted: July 09, 2016 Published: July 15, 2016

With increasing exploration, production and transportation of petroleum in the world, increased the occurrence spill from accidents in the marine environment [4,5] resulting in major impacts [6]. Marine organisms exposed to petroleum contamination, both acute and chronic, shows changes in all levels of biological organization (biochemical, physiological and behavioral changes) [7-13]. Among the marine organism, we finds the *Rachycentron canadum* (Linnaeus, 1766), belongs to Rachycentridae family. Its occurrence is in the Atlantic and Pacific Ocean areas. It classed as inshore and epipelagic species of active swimming habits, due to lack of swim bladder and migratory behavior. It feeds on zoobenthos and nekton, including fish, crustaceans and molluscs, as well as debris left by large animals such as sharks, rays and turtles. Due to this outstanding position in the food chain, this organism can provide important information directly related to the ecosystem in which it lives [14]. In addition, a large commercial interest due mainly to its rapid growth, captive adaptability and excellent quality of their meat [15].

One of the most affected organ is the liver, used with biomarkers [7,16], and it is the most studied in ecotoxicology [17]. The liver is an organ responsible for detoxification and is essential for the metabolism and excretion of toxic substances in the body [18]. Exposure to water-soluble fraction of petroleum can cause histological alterations in the liver. Brusle and Anadon [19] claim that fish liver histology could serve as a model for studying the interactions between environmental factors and liver functions and structures. Another important factor is the investigation of liver enzymes. The main enzymes used for diagnosing liver lesions are alanine aminotransferase (ALT) and aspartate aminotransferase (AST) this because when the activity of these enzymes is determined in the serum indicates that the liver was damaged and the enzyme was extravasated for serum [1].

The aim of this paper was to evaluate the liver histological changes, hepatosomatic index and condition factor, as well as, the activity of the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) after sublethal exposure to water-soluble fraction (WSF) of petroleum for 7 and 14 days.

Materials and Methods

Extraction of water-soluble fraction (WSF) of petroleum

Crude petroleum sample were donated by the Petrochemical Center of Petrobras, Maritime Terminal Almirante Barroso, São Sebastiao Sao Paulo Brazil and characterized chemically and physically.

In a ratio of 1:10 (v / v), crude petroleum was mixed with sea water and, through ultrasonic waves, the water-soluble fraction (WSF) of petroleum was separated during 60 minutes (State Oceanic Administration, 2007). WSF was analyzed by spectrofluorometer SPEX Fluorolog 1681 (Spex Industries, Inc., Edison, NJ) to determine the concentration of polycyclic aromatic hydrocarbons (PAH). To determine the concentration of PAH from the water-soluble fraction in petroleum water comparing with sea water, the samples were excited at the wavelength range of 200 to 600 nm, with $\Delta\lambda$ set at 10 nm intervals and confirmed by a Horiba OCMA 350 automatic analyzer (Horiba Instruments Inc., CA).

Acclimation

Amounting to a total of 40 individuals of the species *Rachycentron canadum* [20], with total length of 23.18cm (\pm 1.75), standard length of 18.56cm (\pm 1.69) and weight 52.26 g (\pm 3.77), female and male, were obtained from commercial fish farms in Ilha Bela, Brazil and transferred to the biotery aquatic animals of the Evolutionary Histophysiology Laboratory (University of Sao Paulo), where the experiments were conducted.

In semi-static system (the exchange 50% of the water daily), the animals were conditioned in tanks of 150 Liter. The abiotic conditions were controlled daily keeping the following averages: pH (8 ± 0.2), photoperiod (12 day hours/12 night hours), temperature ($23 \pm 2.0^\circ\text{C}$), salinity ($34 \pm 2\%$), dissolved oxygen ($6.15 \pm 0.12 \text{ mg L}^{-1}$) and ammonia concentration ($0.15 \pm 0.05 \text{ mg L}^{-1}$). Fresh fish in pieces was provided *ad libitum* once a day. Under these conditions, the fish underwent an acclimatization period of 15 days to reduce the stress.

Sublethal exposure

The animals were divided into four groups (n 10): two control groups for 7 and 14 days and two experimental groups, exposed to 0.3 ppm WSF for a period of 7 and 14 days. The sublethal concentration was established after obtaining the 50% lethal concentration (LC50) of 0.4 ppm. The abiotic conditions were equal acclimation period (section 2.2). Twenty-four hours before the experiment end, feed was interrupted and sacrificed fish. Protocol approved by the Ethics Commission on the Use of Animals the Institute of Biomedical Sciences, University of São Paulo (protocol n.124, pgs. 109, Book 02, 2011).

Obtaining study material

After the period of exposure sublethal to WSF of 7 and 14 days, fish were anaesthetized with benzocaine 2%, and punctured the blood from the tail vein and sacrificed after spinal cord section. The procedures were submitted for approval by Committee Ethics on Animal Use of the Institute of Biomedical Sciences - University of Sao Paulo.

Blood was collected by syringe, without anticoagulant, and transferred immediately to test tubes and packed in ice. Subsequently serum was separated by centrifugation ($2000 \times g$ for 25 min at 4°C , Zentrifugen® Hettich centrifuge) and stored in a freezer at -80°C for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity.

For obtaining somatic indices, the liver of all animals were previously weighed with a precision scale Bel Engineering. Liver samples were fixed in McDowell's fixative and frozen at 4°C (1% glutaraldehyde and 4% paraformaldehyde in pH 7.4 phosphate buffer) [21], for histological analyzes.

Somatic index

Were calculated the condition factor and the hepatosomatic index according to Weibel et al. [22].

Condition factor (CF) was determined from measurements of the length and weight of each sample using the following formula: $\text{CF} = (\text{fish weight} / \text{fish total length}) \times 100$. The hepatosomatic index (HSI) was measured from the weight of the liver and fish according to formula $\text{IHS} = (\text{liver weight} / \text{fish weight}) \times 100$.

Histological analysis

Liver sample was subjected to alcohol dehydration, and then embedded in Paraplast® (Sigma). After the embedment process, were

cut in the microtome (Zeiss® Hyrax M25), with a thickness of $4\mu\text{m}$ (random and non-sequential cut). The slides were mounted and stained with hematoxylin & eosin.

For morphometric analysis of liver were photodocumented 5 random fields of five different sections of each animal, by microscope (Carl Zeiss, Gottingen, Germany) with system photodocumentation AxioCam HRC, at a 1000x, 400x and 100x magnification, totaling 25 photodocumentation per animal for each magnification.

The images were submitted to the program measurement, Image J (National Institutes of Health, NIH) [23]. We analyzed 100 nucleus areas of hepatocytes and counting the number of vessels.

The liver was analyzed semi-quantitatively using Histological Alterations Index (HAI), according to the criteria established by Poleksic and Mitrovic-Tutundzic [24]. The method consists of two criteria, where the first criterion assesses the location and the type of change and the second criterion assesses the stage of severity: stage I, which do not affect the functioning of the organ; stage II, more severe and impair the normal functioning of the organ; and stage III, very severe and irreversible. HAI was calculated using the formula: $\text{HAI} = 1 \times \sum I + 10 \times \sum II + 100 \times \sum III$, with *I*, *II* and *III* corresponding to alterations of stage I, II and III. The average HAI was divided into five categories: 0–10 = normal functioning of the tissue; 11–20 = mild to moderate alteration; 21–50 = moderate to severe alteration; 51–100 = severe alteration; ≥ 100 irreparable alteration.

Activity of protein metabolism enzymes

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were determined by serum, using commercial evaluation kit IDEXX VetTest™ with the assistance of IDEXX VetTest™ equipment.

Statistical analysis

Statistical analysis was conducted using ANOVA followed by Tukey HSD post-hoc comparisons which were performed using SPSS software (SPSS, Release 14.0, SPSS, Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

Results

Somatic index

With the calculation of hepatosomatic index (HSI) and condition factor (CF), observed an average of $3.48 (\pm 0.23)$ and $0.47 (\pm 0.03)$ for control animals 7 days, an average of $7.08 (\pm 0.81)$ and $0.44 (\pm 0.03)$ for animals exposed to WSF within 7 days, an average of $3.47 (\pm 0.40)$ and $0.49 (\pm 0.05)$ for control animals 14 days and an average of $5.69 (\pm 2.28)$ and $0.34 (\pm 0.09)$ for animals exposed to WSF within 14 days, respectively.

It was observed statistical difference in HSI between the group control 7 days and the group exposed to WSF 7 days ($p=0.005$) and between the group control 14 days and the group exposed to WSF 14 days ($p=0.048$). Was observed also a statistical difference in CF between the control group 14 days and the group exposed to WSF 14 days ($p=0.004$) (Table 1).

Histological analysis

Microscopic analysis of liver of the animals in the control groups, described a normal pattern, shown in Figure 1A were observed: cells forming hepatic cords and hepatocytes with homogeneous cytoplasm and well-defined nucleus.

Table 1: Averages (\pm S.D.) of the hepatosomatic index (HSI) and condition factor (CF) of *Rachycentron canadum* exposed to WSF for 7 and 14 days.

Experimental Group	HSI	CF
Control-7 days	3.48(\pm 0.23) ^a	0.47(\pm 0.03)
WSF-7 days	7.08(\pm 0.81) ^b	0.44(\pm 0.03)
Control-14 days	3.47(\pm 0.40) ^a	0.49(\pm 0.05) ^a
WSF-14 days	5.69(\pm 2.28) ^b	0.34(\pm 0.09) ^b

^{a-b}/** Statistical significant differences (p<0.05)

With the morphometric analysis of the nucleus of the hepatocytes, which was obtained an average of 8.29 μm^2 (\pm 1.96) for control animals 7 days, an average of 10.75 μm^2 (\pm 1.93) for animals exposed to WSF for 7 days, an average of 8.34 μm^2 (\pm 1.95) for control animals 14 days and an average of 12.23 μm^2 (\pm 2.89) for animals exposed to WSF for 14 days, causing a significant statistical difference between the control group 7 days and exposed groups to WSF for 7 days (p=0.0001), between the control group 14 days and exposed groups to WSF for 14 days (p = 0.0001) and between the exposed groups to WSF for 7 days and exposed groups to WSF for 14 days (p = 0.0001). Featuring a hypertrophy of nucleus of the hepatocytes.

Were made density analysis of hepatic blood vessels was observed an average of 7.40 blood vessels (\pm 1.71) to the controls animals 7 days, an average of 9.80 blood vessels (\pm 1.75) for animals exposed to WSF for 7 days, an average of 8.30 blood vessels (\pm 1.57) to the controls animals 14 days and an average of 10.50 blood vessels (\pm 1.27) for animals exposed to WSF for 14 days, resulting in a statistically significant difference between the control group 7 days and exposed group to WSF for 7 days (p=0.009) and between the control group 14 days and exposed group to WSF for 14 days (p=0.019).

Were observed further disarrangement of hepatic cords, cell atypia contour, intense cytoplasmic vacuolization (Figure 1B-D) and nuclear and hyperemia (Figure 1C) groups exposed to WSF for 7 and 14 days. With the application of the Histological Alterations Index of liver obtained I = 0 for control groups 7 and 14 days featuring functionally normal organs and I = 24 for animals exposed to WSF for 7 and 14 days featuring organs with moderate to severe alterations.

Activity of protein metabolism enzymes

The aspartate aminotransferase activity (AST) was observed an average 14.25 U/L (\pm 2.75) for control group 7 days, an average 33.00 U/L (\pm 2.94) for experimental group exposed to WSF for a period of 7 days, an average 12.75 U/L (\pm 1.71) for control group 14 days and an average 60.25 U/L (\pm 22.76) for experimental group exposed to WSF for a period of 14 days (Table 2). The data for experimental group exposed to WSF for a period of 14 days was significantly higher compared to the control groups 14 days (p=0.0001), as well as the group exposed to WSF for a period of 7 days compared to exposure 14 days (p=0.027). With the analysis of the alanine aminotransferase activity (ALT), the data obtained in all the groups was <10 U/L, which means that the ALT activity was not detected in the samples (Table 3).

Discussion

The condition factor is often used to study fish biology, as it provides important information about your physiological state, based on the principle that individuals of a certain length, with higher weight are in a better physiological condition [25].

Many studies have used this index as an additional reference for reproduction and seasonal cycle's study, in addition to investigating the effect of pollutants [26-29]. Moles and Norcross observed a

decrease in CF in juvenile *Pleuronectes asper*, *Pleuronectes bilineatus* and *Hippoglossus stenolepis* exposed to sediment contaminated by oil.

In the present study, we observed that the condition factor of animals WSF-14 days group is statistically lower in respect of animals in the control-14 day's group, showing a depletion of physiological conditions. This depression is caused by hydrocarbons which, according Kiceniuk and Khan [30], hamper the absorption of food. Corroborating the study Carls et al., who observed that food contaminated with oil caused reduced growth in *Onchorhynchus gorbuscha*.

The liver can be considered a target organ, it is an essential organ for detoxifying metabolism and excretion of toxic substances [31,32]. The alteration of physical structure and biochemistry of this organ may indicate the state of health of the animals after exposure to pollutants [33].

The hepatosomatic index in fish may indicate liver metabolic status changed [34,35] being a good hepatic parameter. Increased HSI was observed in studies in environments contaminated by polycyclic aromatic hydrocarbons, in *Ameiurus nebulosus* [36,37], corroborating the present study, who observed an increase in the HSI in the group exposed to the WSF for 7 and 14 days.

Increased of the HSI and decreased in CF are observed in studies where animals were collected in contaminated areas [38-40], as observed in this study in animals of the group exposed to the WSF for 14 days. Khan (1998) [41] asserts that the relationship between the increase in HSI and decrease in CF suggests that exposure to WSF of petroleum, not only affects the hepatic metabolism, but also the general physiology of fish.

However, Domingo et al. [42] observed increased HSI with no change in CF, as observed in this study in animals exposed to WSF for 7 days.

These data show that exposure for a period of 7 days did not affect the overall physiology of fish, but it was sufficient to change the hepatic metabolism.

Another hepatic parameter analyzed was histological alteration. Observed an intense cytoplasmic vacuolization in animals exposed to WSF of petroleum. This intense vacuolization can be indicative of degenerative processes, resulting from abnormal metabolism of lipids, such as peroxidation [43,44], possibly as a result of exposure to WSF.

Thus, intense vacuolization may have led to the derangement of hepatic cords observed in animals exposed to WSF for 7 and 14 days. It was observed also, in animals exposed to WSF, nuclear swelling of the hepatocytes, which may indicate an intensification of metabolic activity of hepatocytes [45].

It was noted hyperemia of hepatic vessels in the group of animals exposed to WSF. The hyperemia is the increase of blood cells probably caused by increased blood flow in the liver, thereby facilitating transport of nutrients and improves oxygenation in areas with possible injuries [46].

It was analyzed the activity of AST and ALT enzymes. These enzymes play a key role in the catabolism of amino acids and are used to demonstrate the liver damage [46-48]. AST is an enzyme localized predominantly in mitochondria and ALT is an enzyme localized in

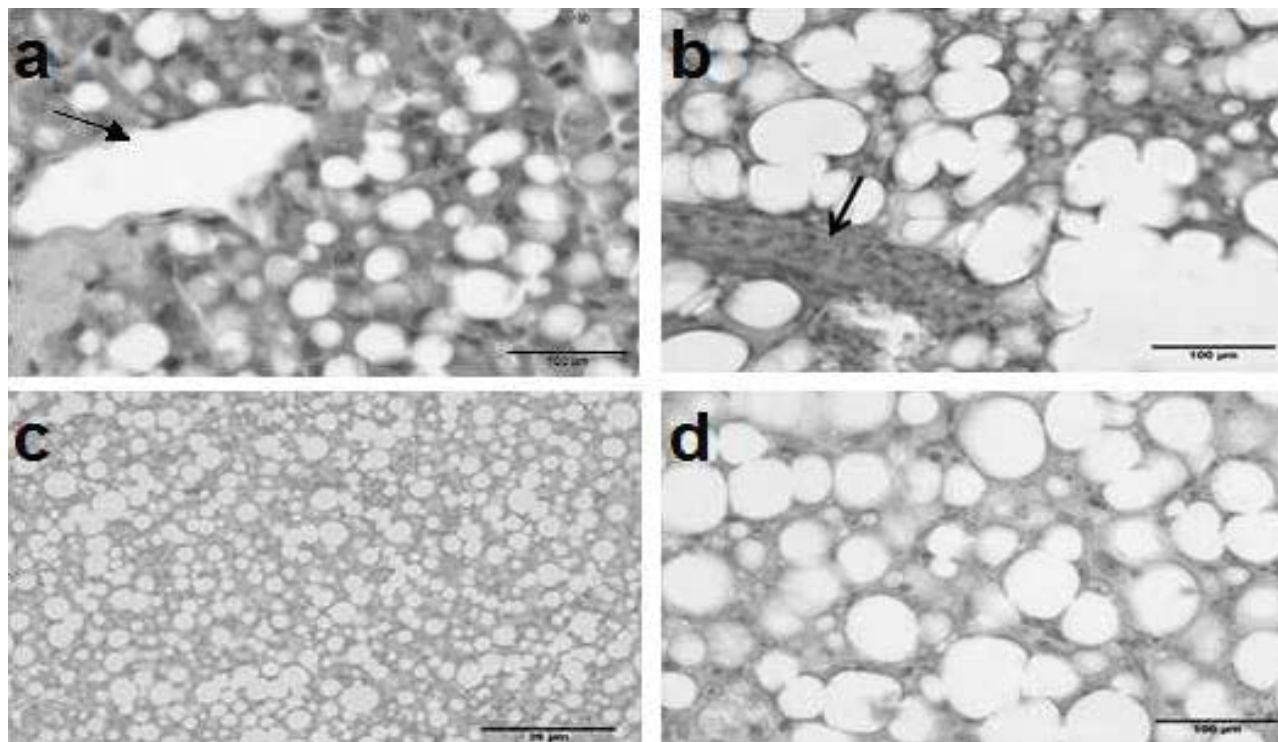


Figure 1: Photomicrograph of histological section in paraffin of the liver of *Rachycentron canadum*

a: Control: The black arrows indicate hepatic blood vessels without accumulation of blood cells; Hematoxylin and eosin staining.
 b-d: Exposed to WSF: Imagem mostra intensa vacuolização citoplasmática; Hematoxylin and eosin staining.
 c: Exposed to WSF: The black arrows indicate hyperemia; Hematoxylin and eosin staining.

Table 2: Histological alterations found on the liver in *Rachycentron canadum* exposed to WSF for 7 and 14 days and their respective Histological Alterations Index (A) represents absence and (P) represents presence of alteration.

Alterations	Stage	Control-7 days	WSF-7 days	Control-7 days	WSF-7 days
Derangement of Hepatic cords	I	A	P	A	P
Atypia cell contour	I	A	P	A	P
Increased of the nuclear volume	I	A	P	A	P
Intense cytoplasmic Vacuolization	I	A	P	A	P
Hyperemia	II	A	P	A	P
Vacuolization nuclear	II	A	P	A	P
Histological Alterations Index		0	24	0	24

Table 3: Averages (\pm S.D.) of the aspartate aminotransferase activity (AST) in U/L of *Rachycentron canadum* exposed to WSF for 7 and 14 days.

Experimental Group	AST(U/L)
Control-7 days	14.25(\pm 2.75)
WSF-7 days	33.00(\pm 2.94) ^b
Control-14 days	12.75(\pm 1.71) ^b
WSF-14 days	60.25(\pm 22.76) ^a
^{a,b} Statistical significant differences (p<0.05)	

the cytosol of liver cells, both with low activity. After a modification of liver cells, the ALT enzyme is released fast in comparison AST enzyme, increasing the activity in the serum [49].

In this study, only it noted the increase in AST activity in serum, suggesting that exposure to WSF the petroleum affected liver cells, impairing their function. However, being a chronic exposure was not detected ALT enzyme activity.

Some studies show changes in AST enzyme activity in fish exposed to other contaminants such as clomazone [50], glyphosate [46,51] and copper [52-58]. For being an organ responsible for many biochemical reactions, including metabolism of hydrocarbons, the liver becomes sensitive to exposure to WSF the petroleum. Once damaged, they can reduce their functions, harming biotransformation system in fish.

It is noteworthy that the evaluation of the effect of exposure to WSF the petroleum in marine fish *Rachycentron canadum* is of utmost importance. The data in this study show that animals have changes in hepatic parameters, may alter vital functions and lead the animals to death. In addition, knowledge of the changes and their adaptations served as the basis for the conservation of the species and more effective environmental monitoring.

Conclusion

It was concluded that the evaluation of hepatosomatic index,

histopathological alterations and activity of enzymes aspartate aminotransferase (AST) are sensitive to exposure to WSF, being good biomarkers. It is concluded, also, that the sub-lethal exposure to 0.3 ppm WSF change of hepatic parameters of the fish species *Rachycentron canadum*, affecting the functioning of the biotransformation system.

Declaration of Interest

Authors thanks Coordenação de Aperfeiçoamento de Pessoal do Nível Superior-CAPES and Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP (2011/18220-1) for the financial support.

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