REPORT

Growth and Histopathological Effects of Chronic Exposition of Marine Pejerrey *Odontesthes argentinensis* Larvae to Petroleum Water-Soluble Fraction (WSF)

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Received: 23 April 2011 / Revised: 12 October 2011 / Accepted: 7 February 2012 / Published online: 29 March 2012

Abstract The water-soluble fraction (WSF) of petroleum contains a mixture of polycyclic aromatic hydrocarbons, volatile hydrocarbons, phenols, and heterocyclic compounds, considered deleterious to aquatic biota. Marine “pejerrey” *Odontesthes argentinensis* (Teleostei: Atherinopsidae) has a great commercial importance in local fisheries and a high potential for aquaculture. The aim of this study was to evaluate the histopathological effects in “pejerrey” larvae exposed to different concentrations of petroleum WSF. The chronic toxicity test was conducted with newly hatched larvae exposed for 21 days to sublethal concentrations of WSF (2.5, 5, 10, and 20 % of WSF), plus one control. Survival and growth were significantly lower in the highest concentration. Several histopathological changes were found in the gills (e.g., hyperplasia, aneurisms, edema, and necrosis), kidney (e.g., nuclear alterations, decrease in the hematopoietic cells), and liver (e.g., hypertrophy, karyorrhexis, and karyopyknosis). An index of branchial lesion was proposed to standardize gill lesions to different pollutants.

Keywords Toxicology · Petroleum WSF · “Pejerrey” · Histopathology · Lesion index

INTRODUCTION

Coastal zones are transition lines between land and sea. They show intense natural dynamics and have been the last depository for a vast array of compounds discharged accidentally or deliberately by humans (Kennish 1997; Clark 2001).

Aquatic pollution caused by petroleum is widely recognized due to its regular use for humanity. Petroleum is known as a complex mixture of many chemicals and the

amount that is soluble in water is generally <1 %. That water-soluble fraction (WSF) of petroleum is a mixture of complex components like hydrocarbons (HCs) and heterocyclic compounds (Zioli and Jardim 2002; Akaishi et al. 2004). Special attention is required to monocyclic aromatic HCs—such as BTEX compounds (benzene, toluene, ethylbenzene, and xylene), which are hydrosoluble and most acutely toxic to aquatic biota—but some attention should be given to polycyclic aromatic HCs (PAHs), which are lipid soluble and tend to bioconcentrate in aquatic animals (Barron et al. 1999).

Previous toxicological investigations with petroleum and refined products have been conducted using histopathology as biomarker, and the use of aquatic organisms as bioindicators and biomonitors of pollution is also well-consolidated in environmental contamination monitoring activities (Wester et al. 2002; Nero et al. 2006).

Different studies have assessed the toxicity of petroleum WSF in fish and they have shown that these organisms are reliable bioindicators of PAHs and BTEX contamination in marine samples. Negative effects of petroleum WSF were reported upon fish growth and metabolism, due to pathologies in organs such as liver, gills, and kidney (Lockhart et al. 1996; Khan 2003; Morales-Caselles et al. 2006; Ramachandran et al. 2006).

When a specific criterion for classification of lesions is applied uniformly, variation between investigators may be reduced (Wester et al. 2002). According to Bernet et al. (1999), the importance of standardized assessments is based on the possibility of quantification of lesions, statistical evaluation, and also comparisons between different studies. The comparison of injury rates may be easier than that between the morphological or pathological descriptions.

Fish are widely used in studies of aquatic toxicology. The choice of species depends on regulatory requirements

(environmental organizations), representativeness, local habits, availability, knowledge, and experience with the species (Di Giulio and Hinton 2008).

The pejerrey *Odontesthes argentinensis* (Valenciennes 1835) (Teleostei: Atherinopsidae) is distributed from São Paulo, Brazil, to the south coast of Argentina. It is an important item in local fisheries, and its introduction has been considered for aquaculture activities (Sampaio 2006). Several studies have been conducted to improve the larviculture of *O. argentinensis*; and this knowledge of larval rearing has also stimulated the establishment of protocols for its use in ecotoxicological assessment (Sampaio and Minillo 1995; Sampaio et al. 2006).

The main goals of this study were to evaluate the acute and chronic toxicity (growth and histopathology) of petroleum WSF on early life stages of marine pejerrey *O. argentinensis* in laboratory experiments. In order to facilitate the interpretation of the effects of branchial histopathology, a lesion index was adapted for the species.

MATERIALS AND METHODS

Sampling and Maintenance of Organisms

Natural fertilized eggs of *O. argentinensis* were collected at Cassino beach (32°30'S, 52°30'W, Rio Grande, RS, Brazil) and immediately transported to the Laboratory of Marine Fish Culture of the Federal University of Rio Grande (FURG). They were incubated in a tank of 50 L with filtered (5 µm) seawater at salinity 30, temperature of 23 °C, and dissolved oxygen ≥ 6.2 mg L⁻¹. Light was continuously provided by fluorescent bulbs at 750 lux. Soon after hatching, larvae were transferred to circular tanks with capacity for 1000 L, with constant aeration and 50 % daily water renewal to maintain the water quality. Larvae were fed with newly hatched *Artemia* sp. nauplii, according to the protocol of larviculture for this species (Sampaio 2006), until 17 days after hatching (DAH) for the acute toxicity test. Newly hatched larvae were used in the chronic toxicity test.

Petroleum WSF Preparation

A Brazilian heavy crude oil (API = 19°) was diluted with filtered seawater (5 µm), and this mixture was placed in a sealed “Mariotti” flask (5 L) with a magnetic stirrer (Quimis, Q241, Brazil), for approximately 22 h at room temperature (25 °C) using a fume hood, without light exposure, according to the method proposed by Anderson et al. (1974). After an hour of resting, the WSF obtained was diluted in different concentrations, using seawater filtered to achieve pre-set concentrations for the toxicity tests.

Toxicity Tests

Acute Toxicity Test

Larvae (17 DAH, 1.9 mg live weight) were exposed semi-statically to six concentrations of petroleum WSF (5, 10, 20, 40, 60, and 100 %), plus a control (without petroleum WSF), during 96 h. All treatments and the control were done in triplicate. Stocking density was 10 larvae per experimental unit (1 L beakers), and food was not provided during the toxicity tests. The beakers were covered with aluminum foil, and aeration was not provided to minimize volatilization of toxicants.

Water quality parameters were measured (temperature, dissolved oxygen, pH, and salinity), 50 % of the experimental media was renewed, and dead larvae were removed and counted. Photoperiod was 12 h L/D, salinity was measured with refractometer (Atago, S/Mill, Japan), pH with an electronic pHmeter (Hanna, 221, Romania), and dissolved oxygen and temperature with a digital temperature/oxygen meter (Yellow Spring International, 55/12 FT, USA).

The median lethal concentration (LC₅₀) was estimated using the software Trimmed–Spearman–Karber method (Hamilton et al. 1977). The safe level was estimated using the LC₅₀ 96 h value and an application factor (10 % of the LC₅₀ 96 h value) proposed by Sprague (1971).

Chronic Toxicity Test

Newly hatched larvae were exposed semi-statically during 21 days to four sublethal concentrations of petroleum WSF (determined in accordance to the acute exposure results): 2.5, 5, 10, and 20 % of petroleum WSF, plus a control group. All treatments and the control were conducted with four replicates; stocking density was 15 larvae per experimental unit (1 L beakers). Physical–chemical parameters were measured (see “Acute Toxicity Test” section), and dead larvae were removed and counted daily.

Larvae were measured (total length) with a stereoscopic microscope and weighed with a balance at the beginning of the test ($n = 5$) and thereafter weekly (n per treatment = 8) until the end of the trial (21 DAH), when all the remaining larvae were weighed and measured. Before measurements, larvae were anesthetized and euthanized with benzocaine (50 ppm), and fixed in Bouin liquid for 12 h and transferred to alcohol 70 % until histological processing (see “Histological Processing” section). Weight gain and specific growth rate (SGR) were calculated to evaluate the growth of pejerrey larvae.

Histological Processing

Larvae were embedded sagittally in paraffin through routine histological processing, sectioned with 7 µm of

thickness and stained with hematoxylin-eosin. Gills, liver, and kidney of all larvae were analyzed under optic microscopy (Olympus BH-2) and photographed with a digital camera.

Qualitative and Quantitative Analyses of WSF

Qualitative and quantitative petroleum HCs were determined in an undiluted sample (1 L) of the original petroleum WSF. The following analyses were performed at ISATEC Laboratory (Rio Grande, Brazil). The 16 priority PAHs ($\Sigma 16$ PAHs) recommended by US-EPA were determined by gas chromatography according to US-EPA Method 8270D (US-EPA 2006), using a Perkin-Elmer (Clarus 500) with a mass spectrometer (MS) detector with an autosampler. The BTEX analysis was taken with 50 mL samples and conducted according to EPA 8015B method (US-EPA 1996), using a Perkin-Elmer (Clarus 500) GC with a flame ionization detector (FID) and a headspace Turbomatrix HS 40 sampler.

Histopathology Evaluation

The protocol proposed by Bernet et al. (1999) was used to evaluate the branchial histopathology of pejerrey larvae after exposure to petroleum WSF. Branchial alterations were classified into three importance factors: (1) minimal pathological importance, the damage is reversible as the exposure to toxicant finishes; (2) moderate pathological importance, the lesion is reversible in some cases, if exposure finishes; and (3) marked pathological importance, the lesion is usually irreversible and leads to partial or complete loss of organ function (Table 1). Every alteration was assessed using a score ranging from 0 to 6, depending on the extent and degree of alteration: 0—unchanged, 2—mild occurrence, 4—moderate occurrence, and 6—severe occurrence. Intermediate values (1, 3, and 5) were also considered.

Using importance factors and score values, the index of branchial lesion (I_G) was calculated as: $I_G = \sum_{rp} \sum_{alt} (aw)$, where rp = reaction pattern, alt = alteration, a = score, and w = importance factor. This index represents the degree of branchial lesion and the higher the index, the greater the degree of damage.

Statistical Analysis

Growth performance and survival as well as physical-chemical parameters and gill lesion index were analyzed using one-way ANOVA for significant differences ($P < 0.05$) among treatments, and when they were found, Duncan's test was performed using the software Statistica 7.0. Data are presented as mean $\pm$ standard error. All data

Table 1 Gill histopathological alterations in “pejerrey” *O. argentinensis* larvae exposed chronically to petroleum WSF and their respective importance factors (adapted from Bernet et al. 1999)

Reaction pattern	Alteration	Importance factor
Circulatory disturbances	Hemorrhage/hyperemia/aneurysm	1
	Inter cellular edema	1
Regressive changes	Respiratory epithelium lifting	1
	Pillar cell rupture	2
	Lamellar aneurysm	3
	Secondary lamellae fusion	3
	Loss of secondary lamellae structure	3
	Nucleares alterations (karyopycnosis/karyorrhexis)	2
	Atrophy	2
Progressive changes	Necrosis	3
	Respiratory epithelium hypertrophy	1
	Interlamellar epithelium hyperplasia	2
Inflammation	Infiltration	2

WSF water-soluble fraction

were analyzed for normality (K–S) and homoscedasticity (Levene Test). Percentage values were arc-sin transformed before ANOVA.

RESULTS

The total concentrations of PAHs and BTEX were estimated at 13.55 and 4843.02 $\mu\text{g L}^{-1}$, respectively, and the sum of petroleum HCs analyzed was calculated at 4856.57 $\mu\text{g L}^{-1}$ (Table 2). The BTEX represented the major compounds in the WSF (94 % of the HC analyzed) and naphthalene represented 1 % of the HC analyzed.

The physical-chemical parameters of the acute toxicity test were measured, and the means were 22.59 ± 0.16 °C (temperature), 5.19 ± 0.65 mg L^{-1} (dissolved oxygen), and 7.86 ± 0.11 (pH).

The LC_{50-96 h} and the respective confidence interval were estimated in 55 % (46.48–65.16) of petroleum WSF, and safe level was calculated as 5.5 % of WSF.

For chronic toxicity test, the mean values of temperature, dissolved oxygen, and pH in the experimental media were 22.7 ± 0.1 °C (temperature), 6.37 ± 0.10 mg L^{-1} (dissolved oxygen), and 7.88 ± 0.04 (pH), respectively. These parameters were not affected by different dilutions of the petroleum WSF.

Larval survival was affected by increasing concentrations of petroleum WSF. After 21 days of exposure, survival, SGR, and weight gain in the highest concentration was significantly different ($P < 0.05$) compared to some of the lowest concentrations of petroleum WSF (Table 3).

Table 2 Concentration of total monocyclic aromatic hydrocarbons (BTEx) and polycyclic aromatic hydrocarbons (PAHs) in the WSF of Brazilian petroleum

Hydrocarbon	Concentration ($\mu\text{g L}^{-1}$)
Benzene	1957.78
Toluene	1845.30
Ethylbenzene	63.21
Xylene	976.73
Total BTEx	4843.02
Naphthalene	9.95
Acenaphthene	nd
Fluorene	nd
Phenanthrene	3.60
Anthracene	nd
Fluoranthene	nd
Pyrene	nd
Benzo[a]anthracene	nd
Chrysene	nd
Benzo[b]fluoranthene	nd
Benzo[k]fluoranthene	nd
Benzo[a]pyrene	nd
Indeno[1,2,3-c,d]pyrene	nd
Dibenzo[a,h]anthracene	nd
Benzo[g,h,i]perylene	nd
Total PAHs	13.55
Sum of petroleum hydrocarbons analyzed	4856.57

nd not detected

Fish exposed to 20 % of petroleum WSF were significantly lower in growth in weight than animals in other treatments (Fig. 1).

The diagnostic of histopathology shows that the gills of pejerrey larvae were affected by exposure to petroleum WSF, the damages were observed at all concentrations of petroleum WSF, and they increased over time, especially at higher concentrations.

The normal structure of the gills can be seen in Fig. 2a, in which the branchial arch supports the primary lamellae

from where the secondary lamellae part. Secondary lamellae are composed of capillaries, pillar cells, and respiratory epithelium. In the control treatment, in all periods analyzed, some abnormalities were observed, such as, epithelial lifting and fusion of secondary lamellae. The lesion index (I_G) was 0.4, 2.6, and 4.9 at 7, 14, and 21 DAH larvae, respectively.

At 2.5 % of petroleum WSF, aneurisms with pillar cell rupture (Fig. 2b), lifting of respiratory epithelium (Fig. 2c), fusion of secondary lamellae, and edema were observed in 14 DAH larvae. 1 week later, a few cases of hyperplasia of interlamellar epithelium were also observed. The I_G determined were 2.8, 16.6, and 15.7 for 7, 14, and 21 DAH larvae, respectively.

With 5 % of petroleum WSF, there was edema, hemorrhage, and lifting of respiratory epithelium, and at 21 days of exposition, other alterations such as hyperplasia of interlamellar epithelium (Fig. 2d), lifting and necrosis of respiratory epithelium, and loss of lamellar structure have occurred. The lesion index for 7 days was 11.4, 12.2 for 14 days and 28.6 for 21 days, respectively.

In the concentration of 10 % petroleum WSF, telangiectasia, fusion of secondary lamellae, loss of lamellar structure, edema, and hemorrhage were observed at 7 and 14 days. The I_G was 24.6 for 7 days and 28.8 for 14 days. It was also observed hyperplasia of the epithelium of the branchial cavity (Fig. 2e). More cases of fusion and loss of secondary lamellar structure were observed at 21 days, and also hypertrophy of respiratory epithelium, in addition to changes already observed previously at 7 and 14 days. The lesion index was 40.0 for 21 days.

The loss of secondary lamellar structure with hemorrhage and nuclear changes (karyopycnosis) was pronounced for the concentration of 20 % of petroleum WSF, with higher occurrence at 21 days of exposition (Fig. 2f). The I_G were 21.4, 38.6, and 60.4 for 7, 14, and 21 days, respectively.

The index of injury to the gill (I_G) showed significant differences among treatments (Fig. 3). For the control, the

Table 3 Survival, weight gain, and SGR of larvae exposed chronically to petroleum WSF (mean $\pm$ standard error)

Treatment	Survival (%)			Weight gain (mg)	SGR (%)
	7 DAH	14 DAH	21 DAH		
Control	98.33 $\pm$ 1.67 ^a	96.66 $\pm$ 1.92 ^a	93.33 $\pm$ 4.71 ^a	10.29 $\pm$ 0.81 ^a	9.22 $\pm$ 0.32 ^a
2.5 %	91.66 $\pm$ 4.19 ^{ab}	90.00 $\pm$ 4.30 ^{ab}	86.66 $\pm$ 3.85 ^a	10.68 $\pm$ 0.4 ^a	9.40 $\pm$ 0.15 ^a
5 %	98.33 $\pm$ 1.67 ^a	95.00 $\pm$ 1.67 ^a	95.00 $\pm$ 1.67 ^a	8.52 $\pm$ 0.87 ^a	8.78 $\pm$ 0.38 ^a
10 %	88.33 $\pm$ 4.19 ^{ab}	83.33 $\pm$ 1.92 ^{bc}	83.33 $\pm$ 1.92 ^{ab}	9.87 $\pm$ 0.81 ^a	9.05 $\pm$ 0.33 ^a
20 %	86.66 $\pm$ 2.72 ^b	71.66 $\pm$ 5.00 ^c	61.66 $\pm$ 9.57 ^b	4.11 $\pm$ 0.72 ^b	5.71 $\pm$ 0.56 ^b

Statistical differences are shown by different letters

DAH days after hatching

WSF water-soluble fraction

Fig. 1 Growth in weight of pejerrey *O. argentinensis* larvae during chronic toxicity test. Statistical significant differences are shown by different letters ($P < 0.05$)

I_G was significantly lower when compared with groups 5, 10, and 20 % of petroleum WSF. For the concentration 2.5 %, the index was lower than 10 and 20 % of WSF. The concentration of 20 % was significantly higher than the control, 2.5 and 5 % of WSF.

The structure of normal liver is shown in Fig. 4a, in which is seen hepatocytes arranged in cords with a single layer of cells lining each sinusoid with energy supplies and uniform nuclei with a single nucleolus.

The liver showed changes according to the increasing of petroleum WSF concentration and the time of exposure, but not as marked as the branchial changes.

Among the most frequent liver alteration, intense cytoplasm basophilia of the hepatocytes and nuclear changes such as hypertrophy, karyorrhexis, and karyopyknosis are described. The nuclear hypertrophy was observed in all concentrations tested and also in the control group, but the incidence was greater in higher concentrations of the petroleum WSF. The other abnormalities described were found only in concentrations of 10 and 20 % of petroleum WSF, such as vacuolar degeneration (Fig. 4b). There were no changes in biliary ducts of livers analyzed.

A normal structure of the anterior kidney at 21 DAH in the control group is shown in Fig. 5a. The kidney is formed by renal tubules, glomeruli, and hematopoietic tissue.

Nuclear alterations were observed in renal tubules, such as hypertrophy, karyorrhexis, and karyopyknosis, in the concentrations of 10 and 20 % (Fig. 5b). Clear spaces between tubular epithelial cells were also observed in the posterior kidney (Fig. 5c), meaning a damage in the structure of the tubules. At these concentrations, less hematopoietic tissue was observed, possibly due to multiple hemorrhages in the gills and constant replenishment of blood cells.

DISCUSSION

Toxicological results with petroleum WSF are still difficult to compare, because different types of petroleum and different ways to prepare the petroleum WSFs are used without patronizing (Saeed and Al-Mutairi 2000; Singer et al. 2000; Zioli and Jardim 2002). However, Brand et al. (2001) working with North Slope crude oil found the same proportion of BTEX in the WSF, which was found in this study. Likewise, the study of Barron et al. (1999) with petroleum WSF corroborates the concentration of BTEX in this study. These confirmations are substantial because BTEX can be highly responsible for the toxicity of fish, despite their high volatility.

Lethal and sublethal effects have been described as a result of BTEX exposure. Rodrigues et al. (2010), working with newly hatched larvae of *O. argentinensis*, found that the median $LC_{50-96\text{ h}}$ value was around 70 % of the Brazilian petroleum WSF, differently from the present investigation in which the value for $LC_{50-96\text{ h}}$ was 55 % of the petroleum WSF for larvae with 17 DAH. This difference may be due to greater contact between the newly formed gills and petroleum WSF. These results are in accordance of different studies, for example, the $LC_{50-96\text{ h}}$ value for *Menidia beryllina* silversides were calculated between 32 and 88 % of the WSF of three different crude oils (Neff et al. 2000).

Survival was significantly lower in the highest concentration of petroleum WSF, as expected, because this concentration is higher than the safe level obtained in the previous acute toxicity test. However, survival in the lowest concentrations (control and 2.5 % of WSF) were slightly lower than survival in the concentration of 5 % of WSF, though there was no statistical differences among these concentrations. This can be due to the genetic differences and health conditions of the eggs and larvae collected in the environment, which is a natural factor and should be taken into account in the case of a real oil spill.

According to Di Giulio and Hinton (2008), fish growth is reduced by exposure to pollutants, because the food consumption decreases or energy expenditure increases due to costs to avoid exposure, for detoxification, or repair tissue. The growth of larvae was lower in the highest concentration of petroleum WSF, as already shown by the SGR and weight gain. This probably was due to the maintenance of the vital functions under stressing conditions, which require energy that could be saved to the development of the organism. Al-Yakoob et al. (1996) also found lower SGR values in the highest petroleum WSF concentrations for the marine fish *M. beryllina*. Carls and Rice (1990) reported slower development, reduced hatching success, survival and growth, and also morphological abnormalities in larvae of walleye Pollock *Theragra*

Fig. 2 Gill sections from pejerrey *O. argentinensis* larvae. **a** Branchial arches (ga) of a larva of 21 DAH in the control group, with primary (pl) and secondary (sl) lamellae with normal structure, $\times 400$. **b** Secondary lamellae with aneurism (arrows) in a larva of 14 days exposed to 2.5 % of petroleum WSF, $\times 400$. **c** Secondary lamellae showing lifting of the respiratory epithelium (arrow) in 21-day old larvae exposed to 2.5 % of petroleum WSF, $\times 1000$. **d** Hyperplasia of

interlamellar epithelium (arrows) in 21-day old larvae in the concentration of 5 % of petroleum WSF, $\times 400$. **e** Branchial cavity of a larva of 14 days exposed to 10 % of petroleum WSF, showing hyperplasia of the cavity epithelium (arrow); pg pseudobranchia, $\times 200$. **f** Gill with a hemorrhage (arrow) in a larva of 14 days exposed to 20 % of petroleum WSF; e esophagus, $\times 200$. Staining: H&E

Fig. 3 Index of injury to the gill (I_G) over the 21 days of the chronic toxicity test with pejerrey *O. argentinensis* larvae. Different letters show significant differences ($P < 0.05$)

chalcogramma, exposed to WSF of a crude oil during the embryos stage. Yolk and body lengths of larvae that hatched from exposed eggs were significantly reduced by increasing concentrations of WSF.

Tissue damage has been reported in animals exposed to BTEX, and it seems that gills deserve more attention because this vital organ is in direct contact with the external environment. For this reason, an index of injury to the gill (I_G) was adapted from Bernet et al. (1999) as an important tool of lesions classification to be applied in petroleum or other pollutant investigation. According to Bernet et al. (1999), the importance of standardized assessments is based on the possibility of quantification of lesions, statistical evaluation, and also comparisons

between different studies. The comparison of injury rates may be easier than that between the morphological or pathological descriptions. This index of injury includes an importance factor, i.e., how the lesions affect an organ function and the ability of the fish to survive. When the damage causes loss of the structure combined with the partial or total loss of the function of an organ or system, it is considered irreversible, even if the stressor is neutralized, and may cause the death of the organism.

In the present investigation, a greater number and more severe lesions were found in the gills of fish exposed to higher concentrations of petroleum WSF. Thus according to the branchial index proposed, the higher the I_G value, the greater the degree of damage in the gills of the larvae, which can be associated to the mortality and lower growth of most affected larvae.

Although there was no significant difference in growth (weight gain), SGR, and survival in lower petroleum concentrations, I_G increased progressively and significant differences among different concentrations were observed. Histological changes appear as medium-term response to sublethal stressors, and seem to be an endpoint of cellular/subcellular changes, however, they may not reflect significant differences in growth or survival in short/medium periods of time. After the tissue level, the lesions can damage an entire organ, system, or even the whole body, causing its long-term death (Bernet et al. 1999; Di Giulio and Hinton 2008).

Fish exposed to petroleum WSF and refined products can exhibit alterations in the gills, such as lifting of respiratory epithelium, tissue necrosis, hyperplasia, hypertrophy, hemorrhage, and telangiectasia (Brand et al. 2001; Akaishi et al. 2004; Rodrigues et al. 2010). Some of

Fig. 4 Liver from pejerrey *O. argentinensis* larvae. **a** Liver of a larva of 21 days in the control group, with lipid reserves (empty arrow), hepatocytes (h) with normal nuclei (arrowhead) and sinusoids (dark arrows), $\times 400$. **b** Liver of a larvae after 21 days of exposure to 20 %

of WSF in which hepatocytes (h) can be observed, with abnormalities in their nuclei (hypertrophy and karyopycnosis, arrow), and also enlarged sinusoids (s), $\times 400$. Staining: H&E

Fig. 5 Kidney sections of *O. argentinensis* larvae. **a** Anterior kidney of a larva of 21 days in the control group, with the renal tubules (t), a glomerulus and hematopoietic tissue (ht), $\times 400$. **b** Anterior kidney of a larva of 21 DAH exposed to 20 % of petroleum WSF. An enlargement of glomerulus (arrow), intercellular spaces (arrowhead), and abnormal nucleus of epithelial cells, $\times 400$. **c** Tubule of the posterior kidney showing hypertrophy (arrowhead) and spaces between cells in a larva of 21 days exposed to 20 % of petroleum WSF, $\times 1000$. Staining: H&E

these lesions are considered irreversible because they cause loss of cell and tissue functions, and after the exposure to toxicant, they can cause death of the organism by imbalances in gill activities such as gas exchanges, ionoregulation, maintenance of acid–base balance, and excretion of nitrogen compounds, mainly ammonia (Di Giulio and Hinton 2008; Duarte et al. 2010).

Romano and Cueva (1988) report that the most common branchial lesion is the respiratory epithelium lifting due to edema, as found in this study. Edema, pillar and endothelial cell ruptures, telangiectasia, and hyperplasia are the result of a reactive and adaptive phenomenon of the epithelium to toxicant exposure. These gill problems lead to the lack of oxygen demanded to normal animal metabolism. Lamellar fusion of gills could be also a protective effect to diminish the gill surface area exposed to the toxicant (Mallat 1985).

Several studies have shown similar deleterious effects of petroleum WSF exposure. Rodrigues et al. (2010) reported epithelial hyperplasia in the gills of *O. argentinensis* larvae acutely exposed to petroleum WSF. Akaishi et al. (2004) reported histopathological effects in the gills of freshwater fish (*Astyanax* sp.), as the disorganization of secondary lamellae and telangiectasia after acute exposure to Campos Bay (Brazil) crude oil WSF. Gills of *Oncorhynchus mykiss* exposed to petroleum WSF for 30 days showed epithelial lifting and damage of secondary lamellae, associated with cellular hypertrophy (Rudolph et al. 2001). Hyperplasia and hypertrophy of the lamellar epithelium also were reported by Khan (2003) in three species of marine flatfishes inhabiting an area near an oil refinery.

Brand et al. (2001) exposed pink salmon fry, *O. gorbuscha*, to sublethal concentrations of Alaska North Slope crude oil WSF for over 10 days and found morphologic and stress-induced lesions in the gills, such as epithelial lifting, fusion, mucous cell hyperplasia, and vascular constrictions. Lockhart et al. (1996) studied the chronic toxicity of Norman Wells crude oil WSF to juveniles of rainbow trout. Harmful effects such as fin erosion and increase of water content in the body were observed.

One of the most common changes in the liver is the hypertrophy of hepatocytes and/or nucleus, although it does not occur not only in stress situations caused by toxicants. The size and volume of hepatocytes reflects its physiological functional status, and in critical periods of fish development, for example, the period of vitellogenesis in adult females these cells can be hypertrophied (Hibiya 1982). As the pejerrey larvae are in a critical period of the development, due to the need for a rapid growth, hypertrophy of hepatocytes can be expected to occur even in the control group. However, it is known that karyocytomegaly

and hepatocyte hypertrophy are intimately related conditions to chronic toxicity (Hinton and Laurén 1990), which can explain the higher occurrence found in larvae exposed to higher concentrations of WSF in the chronic test. The alterations found in the liver of pejerrey larvae have also been reported in the literature to several petroleum compounds (Brand et al. 2001; Stehr et al. 2003; Akaishi et al. 2004; Morales-Caselles et al. 2006; Simonato et al. 2008).

An increased basophilia in hepatocyte cytoplasm can be related to decreased protein synthesis (Sarasquete and Gutiérrez 2005) and possibly to necrotic foci, or may result in loss of energy reserves and rearrangement of cytoplasmic components so that stainable organelles occupy areas that previously contained lipids or glycogen (Hinton and Laurén 1990; Vethaak and Wester 1996). In livers analyzed in this study, several cases of intense basophilia in hepatocytes were also found, which may be related to intense activity of these cells, including detoxification. Morales-Caselles et al. (2006) also reported histopathologies in the liver of *Sparus aurata* juveniles, such as vacuolization of hepatocytes, necrosis and an increased cytoplasmic basophilia related to increase of PAHs.

Akaishi et al. (2004) reported histopathologies in the liver of the freshwater fish *Astyanax* sp., such as presence of necrotic areas and loss of hepatocyte cell limits, increasing with higher concentrations, and also increase of cytoplasm density. Necrosis in the liver is a typical lesion in fish exposed to contaminants; it decreases the number of functional cells in the hepatic tissue with deleterious consequences to the proper functioning of the organ.

The kidney, together with the gills and intestine, are responsible for excretion and maintenance of homeostasis of body fluids. As a large volume of blood flows through the kidney, lesions found in this organ can be useful as signs of environmental pollution (Hinton and Laurén 1990). However, renal changes have been less used as biomarkers of environmental pollution than gills and liver, but are also important biomarkers. Silva and Martinez (2007) reported pathological changes in the kidney of a freshwater “lambari” (*Astyanax altiparanae*) exposed to urban wastewater, similar to those changes found in this study, such as cytoplasmic vacuolation, degeneration, and necrosis in renal tubules, and they also relate these changes to the presence of toxic substances in the filtrate from the glomerulus. Many of these degenerative changes are reversible, and new nephrons can be developed in several weeks after toxicant-induced injury (Hinton and Laurén 1990). In this study, the duration of the chronic toxicity test was not sufficient to observe the development of new nephrons.

The histopathological effects observed in this study can be due to the HCs, demonstrating the usefulness of this tool as a general quality indicator of the aquatic environment (Wester et al. 2002). The importance of using chronic

bioassays is also well-known, because a compound cannot reflect a considerable lethal toxicity, but it is able to produce lesions at different levels to the organism exposed (Morales-Caselles et al. 2006).

CONCLUSION

This study has shown that sublethal exposure to the petroleum WSF causes a significant stress response as indicated by altered structure of gill tissues, as shown by the highest I_G in the concentration of 20 % of WSF, and also by lesions in the liver and kidney. Survival, growth, and SGR were significantly lower in the highest concentrations of the WSF, confirming that pejerrey larvae can suffer irreversible damage when exposed to toxic concentrations of petroleum WSF. The adapted gill lesion index supported the interpretation of the gill damage for chronic toxicity test. Furthermore, *O. argentinensis* larvae may possibly be a bioindicator or biomonitor species of environmental pollution in coastal areas.

Acknowledgments The authors would like to gratefully acknowledge the Brazilian National Agency of Petroleum (ANP) for supporting the students E.P. Gusmão, R.V. Rodrigues, and C.B. Moreira. K.C. Miranda-Filho thanks CNPq (481613/2007-2) and CAPES for their support. L.A. Sampaio is a research fellow of Brazilian CNPq (308013/2009-3).

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