UNIVERSIDADE FEDERAL DO RIO GRANDE
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EFEITO AGUDO E AVALIAÇÃO HISTOPATOLÓGICA DE LARVAS DO PEIXE-REI MARINHO *Odontesthes argentinensis* EXPOSTAS À FRAÇÃO SOLÚVEL DO PETRÓLEO, DIESEL E GASOLINA EM ÁGUA

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RIO GRANDE, RS
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Universidade Federal do Rio Grande
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Dissertação apresentada como parte dos requisitos para a obtenção do grau de mestre em Aqüicultura no programa de Pós-Graduação em Aqüicultura da Universidade Federal do Rio Grande.

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Dedico este trabalho as pessoas mais importantes da minha vida: MEUS PAIS!!!

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RESUMO GERAL

Os hidrocarbonetos de petróleo são considerados um dos principais poluentes dos meios hídricos. No entanto, são escassos os estudos referentes a toxicidade destes compostos sobre os estádios iniciais de desenvolvimento em peixes nativos do Brasil. O objetivo deste trabalho foi avaliar a concentração letal mediana (CL_{50}-96h) e os efeitos histopatológicos da fração solúvel (FSA) do petróleo, do diesel e da gasolina em água, sobre larvas do peixe-rei marinho *Odontesthes argentinensis*. Para a realização dos testes toxicológicos agudos foram utilizadas as seguintes concentrações: petróleo brasileiro (5%, 10%, 25%, 50%, 75%, e 100% de FSA), diesel (1%, 2%, 4%, 8%, 16%, 32%, e 64% de FSA) e gasolina (1%, 2,5%, 5%, 10%, e 20% de FSA), mais a adição do controle para cada poluente. Todos os tratamentos foram realizados com três repetições e com 30 larvas. Após a realização dos testes de toxicidade (96 h), três larvas de cada concentração foram coletadas para estudo histológico. A FSA do petróleo apresentou uma CL_{50}-96h igual a 70,68% (65,73–76,01), enquanto o diesel e a gasolina apresentaram os valores de CL_{50}-96h de 13,46% (10,19-17,79) e 5,48% (4,85-6,20), respectivamente. A avaliação histológica das larvas de *O. argentinesis* expostas a FSA do petróleo, diesel e gasolina mostrou lesões nas brânquias, pseudobrânquias, esôfago e fígado. As brânquias, pseudobrânquias e o esôfago apresentaram hiperplasia em seus epitélios, enquanto o fígado apresentou dilatação do sinusóide hepático, hepatocitomegalia, binucleação e degeneração nuclear dos hepatócitos, assim como núcleos picnóticos. O presente trabalho demonstrou que o diesel e a gasolina são pelo menos cinco vezes mais tóxicos que o petróleo brasileiro, no entanto, todos os poluentes induziram a moderadas lesões histopatológicas nas larvas de *O. argentinensis*.

2. ABSTRACT

The hydrocarbon of petroleum is currently considered as one of the main pollutants of water resources and cause recognized toxicity to aquatic biota. However, there are few studies regarding the toxicity of these compounds on the early stages of development in Brazilian fish. The objective of this study was to evaluate the median lethal concentration (LC_{50}-96h) and the histopathological effects of water-soluble fraction (WSF) of oil, diesel and gasoline, on larvae of marine pejerrey *Odontesthes argentinensis*. During the experiments the concentrations tested were: Brazilian crude oil (5%, 10%, 25%, 50%, 75%, and 100% of WSF), diesel (1%, 2%, 4%, 8%, 16%, 32%, e 64% of WSF) and gasoline (1%, 2.5%, 5%, 10%, and 20% of WSF) plus a control for each pollutant. All treatments were performed with three replicates and 30 larvae. After the toxicity tests (96 h), three larvae of each concentration were collected for histological evaluation. The petroleum presented a median lethal concentration (LC_{50}-96h) equal to 70.68% (65.73-76.01), while diesel and gasoline presented the LC_{50}-96h values of 13.46% (10.19-17.79) and 5.48% (4.85-6.20), respectively. The histological evaluation of *O. argentinensis* larvae exposed to WSF of petroleum, diesel and gasoline showed lesions in the gills, pseudobranchs, esophagus and liver. The gills, pseudobranchs and esophagus showed epithelial hyperplasia, while the liver presented dilation of hepatic sinusoids, hepatocitomegaly, hepatocytes bi-nucleated and nuclear degeneration of hepatocytes, as well as piknotic nuclei. The present investigation demonstrated that diesel and gasoline are at least five times more toxic than Brazilian petroleum, however all toxicants induced to moderate histopathological injuries in pejerrey larvae.

3. INTRODUÇÃO GERAL

Os ambientes aquáticos são considerados os receptores finais de uma grande quantidade de poluentes orgânicos e inorgânicos. Entre os poluentes orgânicos, o petróleo atrai a atenção pública, pois tem sido amplamente utilizado pelo homem como a principal fonte de energia do mundo moderno (Clark 2001). Acidentes envolvendo derramamentos de petróleo e combustíveis derivados ocorrem freqüentemente ao redor do mundo, sendo uma fonte importante de contaminação dos ambientes hídricos. No Brasil, as maiores fontes de acidentes envolvendo estes poluentes seriam: a ruptura de oleodutos e os derramamentos oriundos de navios petroleiros. Outras fontes poderiam ser citadas, tais como os descartes indiscriminados nos grandes centros industriais e urbanos, o que tem gerado um problema crônico de poluição aos ambientes aquáticos (Meniconi *et al*. 2002).

Entre os hidrocarbonetos de petróleo, os hidrocarbonetos policíclicos aromáticos (HPAs) constituem uma das classes de poluentes mais freqüentemente encontradas nos ambientes aquáticos (Kettrup & Marth 1998). Os HPAs representam um grupo amplo de compostos químicos, sendo formados por átomos de H e C e constituídos por até 10 anéis benzênicos (Hylland 2006). Segundo este mesmo autor, existem quatro formas de aporte de HPAs no ambiente marinho: (1) biogênico – produzido por organismos vivos, (2) pirogênico - oriundos de processos de incineração, (3) petrogênico – derivado de combustíveis fósseis e (4) diagênico – derivados de processos de transformação nos solos e nos sedimentos. No entanto, os derramamentos de petróleo e de seus derivados ainda são as principais fontes de poluição dos ambientes aquáticos, tanto dulcícolas quanto os ambientes marinhos e estuarinos (Neff 1985). Todavia, em

alguns casos a principal fonte de toxicidade entre os hidrocarbonetos de petróleo para os organismos aquáticos, não está relacionada aos HPAs e sim aos hidrocarbonetos voláteis monoaromáticos (BTEX - benzeno, tolueno, etilbenzeno e xileno) (Barron *et al.* 1999; Neff *et al.* 2000). Este grupo de hidrocarbonetos são rapidamente absorvidos do meio e causam efeitos deletérios aos organismos aquáticos (Stephens *et al*. 1997).

A fração solúvel em água (FSA) do petróleo e de combustíveis derivados possuem uma mistura de HPAs, BTEX, fenóis, hidrocarbonetos alifáticos e compostos heterocíclicos, contendo nitrogênio e enxofre (Saeed & Al-Mutairi 1999), assim como metais pesados. O emprego da FSA é utilizada em laboratório para avaliar a toxicidade dos hidrocarbonetos de petróleo, tornando também factível, a detecção de efeitos provenientes de acidentes ambientais. De acordo com a origem do petróleo, são encontradas variações na composição química da FSA, que se reflete em diferenças na toxicidade de cada FSA avaliada (Neff *et al*. 2000; Wake 2005). A toxicidade também pode variar em função da metodologia empregada para a preparação e análise laboratorial da FSA (Singer *et al*. 2000; Ziolli & Jardim 2002). Os mesmos autores relatam que diferentes proporções de petróleo em água, diferentes tempos de agitação, assim como a temperatura, o pH da água e a iluminação empregadas na preparação da FSA influenciam na sua composição química. Também foi comprovado que a salinidade da água empregada na preparação da FSA influencia a sua composição final (Ramachandran *et al*. 2006).

Diversos estudos têm demonstrado que a FSA de produtos refinados do petróleo (diesel, gasolina, "bunker") são mais tóxicos que a FSA do petróleo: Anderson *et al.* (1974) demonstraram que a FSA do diesel e o "bunker C" são mais tóxicos que a FSA do

petróleo para peixes e crustáceos, enquanto que Rayburn *et al*. (1996) e Dede & Kaglo (2001) observaram que o diesel apresenta valores de concentração letal mediana (CL_{50}) inferiores aos normalmente encontrados na literatura com relação ao petróleo.

As FSA's do petróleo e de combustíveis derivados vêm sendo utilizadas para determinar o efeito tóxico dos hidrocarbonetos na biota aquática. São empregadas tanto em testes de toxicidade crônica (Al-Yakoob *et al.* 1996; Omoregie & Ufodike 2000), quanto em testes de toxicidade aguda (Anderson *et al.* 1974; Coehn & Nugegoda 2000; Mohammed 2005).

Os peixes são ótimos biomarcadores para avaliar os níveis de contaminação dos hidrocarbonetos de petróleo, pois estes tendem muitas vezes a se concentrar e se acumular mais nos organismos do que no próprio ambiente em função do seu caráter lipofílico (Anyakora *et al.* 2005). Os estádios iniciais de desenvolvimento dos peixes são particularmente sensíveis aos xenobióticos. Ovos e larvas de peixes são muito utilizados em testes de toxicidade com o objetivo de determinar as concentrações legalmente aceitáveis para alguns poluentes no ambiente aquático, assim como determinar os seus efeitos letais e subletais sobre a biota aquática (von Westernhagen 1988). Segundo Stephens *et al*. (1997), os peixes na fase de desenvolvimento larval são normalmente mais sensíveis que os peixes na fase adulta, principalmente pela maior relação superfície/volume, podendo contribuir com um maior influxo de hidrocarbonetos de petróleo para o organismo. Outra característica que potencializa a toxicidade destes compostos, é a reduzida capacidade de locomoção das larvas, quando comparadas aos indivíduos adultos. Contudo, os estudos toxicológicos utilizando o petróleo brasileiro e combustíveis derivados para larvas de peixes nativos do Brasil são inexistentes.

A exposição de peixes aos hidrocarbonetos de petróleo pode gerar:

1. Interferências no sistema reprodutor e anormalidades durante o desenvolvimento de embriões e larvas (Knutzen 1995; Middaugh *et al*. 1998; Carls *et al*. 1999;);
2. Alterações nas funções cardíacas, respiratórias e comportamentais (Widdows & Johnson 1988);
3. Efeitos imunotóxicos (Fossi *et al*. 1997);
4. Formação de "off-flavor" em organismos mantidos em sistemas de criação (Lovell 1983);
5. Efeitos genotóxicos, mutagênicos e carcinogênicos (Carls *et al*. 1999; Baršienė *et al*. 2007; Vanzella *et al*. 2007);
6. Lesões histopatológicas nos tecidos dos sistemas respiratório, digestório e excretor (Lee & Page 1997; Brand *et al*. 2001; Akaishi *et* al. 2004; Simonato *et al*. 2008).

Atualmente, a poluição que atinge os mais variados ambientes pode ser mensurada a partir dos efeitos gerados à biota. Vários são os biomarcadores utilizados para determinar os efeitos tóxicos gerados por poluentes em animais marinhos. Entre estes os mais utilizados estão os biomarcadores bioquímicos, moleculares e histológicos (Stephens *et al*. 1997; Bernet *et al*. 1999; Simonato *et al*. 2008). A histologia é um método rápido para detectar o efeito de diferentes poluentes em vários tecidos e órgãos dos peixes (Bernet *et al*. 1999).

As brânquias e o fígado são órgãos que podem atuar como biomarcadores histológicos para a toxicidade de hidrocarbonetos de petróleo (Brand *et al*. 2001). As

brânquias possuem destacada importância por apresentarem uma fina e elevada superfície de troca, sendo responsáveis por funções metabólicas vitais para os peixes, tais como respiração, osmorregulação e excreção de amônia (Moyle & Cech Jr. 1988). Este órgão possui permanente contato com o ambiente podendo refletir a sua integridade. Em casos de contaminação ou de poluição, as brânquias são uma importante via de entrada dos hidrocarbonetos de petróleo em animais aquáticos (Hylland 2006). Já o fígado destaca-se por ser o principal órgão de desintoxicação em vertebrados e atua através de processos de transformação enzimática de compostos xenobióticos que são concentrados ou ingeridos pelos animais. Por esta razão, o fígado responde rapidamente à presença de poluentes, demonstrando prontamente alterações estruturais, bioquímicas e moleculares (Romano 1999).

Várias espécies de peixes foram padronizadas por agências ambientais para realização de testes laboratoriais de toxicidade. Algumas espécies da família Atherinopsidae são utilizadas em testes de toxidade, como é o caso de *Menidia beryllinia* (Anderson *et al.*, 1974; Hemmer *et* al. 1992; Al-Yakoob *et al*., 1996), *Atherinops affinis* (Hemmer *et al.* 1992), *Odontesthes bonariensis* (Carriquiroborde & Ronco 2006), entre outras. Os exemplares deste grupo de peixes são considerados um bom modelo para testes ecotoxicológicos, pois em geral possuem um pequeno tamanho, são facilmente obtidos e mantidos em laboratório.

O peixe-rei *Odontesthes argentinensis* também pertence à família Atherinopsidae e possui distribuição marinha e estuarina, desde o estado de São Paulo no sudeste do Brasil até o sul da Argentina (Brian & Dryer, 2006), sendo um importante recurso pesqueiro nestas regiões. Nos últimos anos, esta espécie vem sendo alvo de estudos para

sua introdução na aqüicultura. Vários aspectos relacionados à sua criação já foram estudados, entre eles, a sua reprodução, larvicultura e manutenção de juvenis em laboratório (Phonlor & Sampaio 1992; Tesser & Sampaio 2001; Sampaio 2006).

O peixe-rei *O. argentinensis* vem sendo considerado um bom exemplar para testes de ecotoxicidade e já foi utilizado na avaliação toxicológica de compostos nitrogenados (Sampaio & Minillo 1995, 2000; Sampaio *et al.* 2006). Todavia, até o momento esta espécie foi pouco empregada em testes de toxicidade com outros poluentes.

Este trabalho foi realizado com o objetivo de avaliar os efeitos letais e as histopatologias causadas pela FSA do petróleo, diesel e gasolina em água sobre larvas do peixe-rei marinho *O. argentinensis*.

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ARTIGO ANEXO

Efeito agudo e avaliação histopatológica de larvas do peixe-rei marinho *Odontesthes argentinensis* expostas à fração solúvel do petróleo, diesel e gasolina em água

Co-autores: Kleber Campos Miranda-Filho, Emeline Pereira Gusmão, Cauê Bonucci Moreira, Luis Alberto Romano e Luís André Sampaio

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Acute toxicity and histopathology evaluation in newly hatched larvae of marine pejerrey *Odontesthes argentinensis* exposed to water-soluble fraction of petroleum, diesel and gasoline

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Abstract

Acute toxicity of water-soluble fraction (WSF) of petroleum (Brazilian crude oil), diesel and gasoline to newly hatched larvae of marine pejerrey *Odontesthes argentinensis* were studied. Larvae were exposed during 96 h to different concentrations of WSF of petroleum (5, 10, 25, 50, 75, and 100%), diesel (1, 2, 4, 8, 16, 32 and 64%), and gasoline (1, 2.5, 5, 10, and 20 of WSF), plus a control to each pollutant. All treatments were replicated 3 times and 30 larvae were exposed to each concentration. After 96h of exposure to the different WSF, three larvae were sampled for histopathological studies. The increase the WSF of petroleum, diesel and gasoline reduced significantly ($P<0.05$) pH and dissolved oxygen concentration in the water. The median lethal concentration and the respective confidence interval after 96 h (LC_{50}-96 h) of exposure for petroleum was equal to 70.68% (65.73 - 76.01), it was significantly higher ($P<0.05$) than the values for diesel and gasoline, which were 13.46% (10.19 - 17.79) and 5.48% (4.85 - 6.20), respectively. The histological examination of *O. argentinensis* larvae exposed to WSF of petroleum, diesel and gasoline after 96 h revealed a variety of lesions in gills, pseudobranchs, esophagus and liver tissues. The gills, pseudobranchs and esophagus presented epithelial hyperplasia, and liver presented dilatation of hepatic sinusoids, hepatocitomegaly, bi-nucleated and nuclear degeneration of hepatocytes, such as piknotic nuclei. The present work demonstrated that acute toxicity of diesel and gasoline is at least fivefold higher than Brazilian petroleum for larvae of *O. argentinensis*. However, all toxicants induced moderate histopathological abnormalities in pejerrey larvae.

Introduction

Accidents involving petroleum hydrocarbons spills occur frequently around the world, and they are important sources of marine and coastal pollution. In Brazil, major oil related pollution events are associated to the rupture of oil ducts and spills from oil tankers (Meniconi et al. 2002). The water-soluble fraction (WSF) of crude oil and their derivatives contains a mixture of polycyclic aromatic hydrocarbons (PAH), BTEX (benzene, toluene, ethylbenzene and xylenes), phenols and heterocyclic compounds, containing nitrogen and sulfur (Saeed and Al-Mutairi 1999), and also heavy metals. The accumulation of soluble petroleum hydrocarbons in fish is extremely rapid, and these compounds are lipophilic and potentially toxic to aquatic biota (Gravato and Santos 2002).

Fish can be used as bioindicators and biomonitors 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 (von Westernhagen 1988). According to Stephens et al. (1997), larval stages are more sensitive than adult fish mainly because their relative large surface area contributes to higher uptake of petroleum hydrocarbons. The toxicity of these compounds in fish embryos and larvae is commonly due to tissue injury or enzyme inhibition (Pollino and Holdway 2002; Akaishi et al. 2004) and several effects have been reported like malformations, genetic damage, lower growth rate, and high mortality (Carls et al. 1999).

Biochemical, physiological and histological biomarkers, among others, have been used to determine the effects of petroleum 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 marine pejerrey *Odontesthes argentinensis* (Teleostei: Atherinopsidae) is being considered as a new species for aquaculture and ecotoxicological investigations (Sampaio 2006; Sampaio et al. 2006). This species is distributed in coastal and estuarine waters from Southern Brazil to Argentina (Brian and Dryer 2006), being an important fishery resource in these areas. Petroleum industry, mainly refineries, is an important activity in this region, demanding the assessment of their possible deleterious effects on local animals and ecosystems. Other Atherinopsidae fish, like *Menidia beryllina* and *Atherinops affinis*, have already been used in ecotoxicological studies, including petroleum (Anderson et al. 1974; Hemmer et al. 1992; Al-Yakoob et al. 1996).

The toxicity of several petroleum and their derivatives have been studied for different species of fish larvae (Stephens et al. 1997; Neff et al. 2000; Pollino and Holdway 2002), but no information is available about the toxicity of Brazilian petroleum and derivatives with indigenous fish larvae. The aim of this work was estimate the median lethal concentration in four days (LC_{50}-96h), and investigate histological abnormalities caused by WSF of Brazilian petroleum, diesel and gasoline for newly hatched larvae of marine pejerrey *O. argentinensis*.

Material and Methods

1. Biological sampling

Fertilized eggs of marine pejerrey were collected at Cassino Beach (32°30'S, 52°30'W, Rio Grande-RS, Brazil) and transported to the Laboratory of Marine Fish Culture of the Federal University of Rio Grande. They were incubated in a 1,000 L fiberglass tank, filled with filtered marine water (5 µm) at temperature of 23ºC, salinity 30 ‰, and dissolved oxygen concentration ≥ 6.2 mg/L. Light was continuously provided by fluorescent bulbs at 750 lux.

2. Preparation of water-soluble fractions (WSF)

Heavy petroleum was donated by the Brazilian National Petroleum Agency. Diesel and gasoline were obtained directly from a commercial gas station. Water-soluble fractions of petroleum and derivatives fuels were prepared in a fume hood, free of light, mixing slowly one part of toxicant with four parts of saltwater employing a magnetic stirrer (Quimis, Q241, Brazil), during approximately 22 h. The WSFs were produced in a 5 L "Mariotti" flask, in ambient temperature and salinity 30, according to methodology described by Anderson et al. (1974) with some modifications. After 1-2 h of resting, the different WSFs were ready to be used in the toxicity tests.

3. Acute toxicity tests

Acute toxicity tests were carried out with newly hatched larvae during 96 h. Toxicity trials were run in 600 mL beakers filled with 300 mL of test solutions in a semi-static system. Toxicants were replaced 50% daily. During the experiment photoperiod was 12 h L/12 h D and light intensity at the water surface of the experimental units ranged from 750 to 1.000 lux (Chauvin Arnoux, CA 810, France). Temperature and

salinity were kept at 22.8±0.2 °C and 30.3±0.2 during the tests. Throughout the experiment, oxygen (Yellow Spring International, 55/12 FT, USA) and pH (Hanna, 221, Romania) were measured daily. Aeration and food were withheld during the toxicity tests.

Preliminary tests were performed in order to define the lethality range for each toxicant. Definitive concentrations (experimental solution containing a percentage of WSF were: petroleum (10, 25, 50, 75, and 100%); diesel (4, 8, 16, 32, and 64%); gasoline (1, 2.5, 5, 10, and 20%), plus a control without addition of hydrocarbons. All treatments and controls were conducted in triplicate, where 30 fish were randomly distributed for each treatment (n=10 per flask). Fish mortality was observed every 24 h and larvae were considered dead when they remained immobile on the bottom of the beakers, even after mechanical stimuli with the tip of a glass pipette.

4. Hydrocarbons analysis

Total hydrocarbons analyses were determined for 100% WSF of petroleum, diesel and gasoline. The PAHs were analyzed according to EPA 8270D method, using a Perkin-Elmer (Clarus 500) gas chromatograph with a mass spectrometer (MS) detector with an autosampler. The BTEX were analyzed according to EPA 8015B method, using a Perkin-Elmer (Clarus 500) gas chromatograph with a flame ionization detector (FID) and a headspace Turbomatrix HS 40 sampler.

5. Histological analysis

At the end of the acute exposure to the different WSFs, three larvae of each experimental unit were sampled for histological studies. Larvae were euthanized with benzocaine 30 ppm and fixed in Bouin's liquid. Whole-larvae were dehydrated in a

graded series of ethanol, embedded in paraffin, sectioned (7µm) and the slides were stained with hematoxylin and eosin. Slides were examined by light microscopy (Olympus BH-2 microscope) and the images were registered with a digital camera.

6. Statistical analysis

Median lethal concentration (LC_{50}-96h) for each toxicant and their respective confidence intervals (95%) were calculated using the software Trimmed Spearman Karber method (Hamilton et al. 1977), and the safe levels were estimated according to Sprague (1971). Comparisons among water quality parameters and LC_{50}-96h of petroleum, diesel and gasoline WSFs were calculated using one-way ANOVA, followed by the test of Tukey, with significance level of 95%. The software Statistica 6.0 was used for all analysis.

Results

The HPAs and BTEX present in WSF of petroleum, diesel and gasoline are summarized in Table 1. The WSF of diesel and gasoline presented high concentrations of BTEX and low concentrations of PAHs, while the WSF of petroleum presented high concentrations of PAHs specially naphthalene and low concentrations of BTEX.

The dissolved oxygen concentration and pH were significantly reduced by the WSF of diesel, and gasoline. There was no influence of WSF of petroleum on pH, but the dissolved oxygen concentration was also reduced. The lowest pH values were observed at the highest WSF of diesel (7.71) and gasoline (7.56), significantly lower ($P<0.05$) than the controls, which were equal to 8.12. Overall dissolved oxygen concentration for WSF of petroleum, diesel, and gasoline was 6.3 mg/L in the controls. However, increasing

concentration of hydrocarbons led to significant reduced ($P<0.05$) levels of dissolved oxygen concentration, which were equal to 5.20, 3.71, and 3.36 mg/L for 100% WSF of petroleum, 64% WSF of diesel, and 5% WSF of gasoline, respectively.

No mortalities were observed after 24 h of exposure of larvae exposed up to 75% WSF of petroleum. However, 76.7% of the larvae exposed to 100% WSF of petroleum died within the first 24 h of exposure. The lowest concentration where dead larvae were observed was at 50% WSF of petroleum after 96h of exposure. At the same time 53.3% of mortality was observed for larvae kept at 50% WSF of petroleum (Table 2).

High mortality (90%) was observed for larvae exposed to 32% WSF of diesel within the first 24 h of exposure, but only 3.3% of larvae exposed to 16% WSF of diesel died at the same time, while no mortalities were registered at lower concentrations. After 96 h of exposure there were no dead larvae exposed to concentrations below 16% WSF of diesel, accumulated mortality raised to 6.7% at 16% WSF of diesel, and all larvae exposed to 64% WSF of diesel were already dead within the first 48 h of exposure (Table 2).

The WSF of gasoline showed higher toxicity than the WSF of petroleum and diesel. Dead larvae (90%) were already observed within 24 h of exposure to 10% WSF of gasoline, but at lower concentrations it was not observed any mortality. However, next day dead larvae (3.3%) were observed at 2.5% WSF of gasoline. After 96 h no mortalities were observed among larvae exposed to 1% WSF of gasoline, but mortality at 2.5% had raised to 6.7%. Furthermore, all larvae exposed to 10% WSF of gasoline were dead (Table 2).

The LC_{50}-96h of gasoline and diesel were significantly lower ($P<0.05$) than petroleum, but no difference ($P>0.05$) was found between the acute toxicity of gasoline and diesel fuels for newly hatched larvae of *O. argentinensis* (Table 3).

The histological examination of *O. argentinensis* larvae exposed to WSF of petroleum, diesel and gasoline revealed abnormalities on gills, pseudobranchs, esophagus and liver tissues. These alterations were more conspicuous with increasing WSF concentrations. The pseudobranchs showed hyperplasia of the epithelium (Fig. 1B). The major branchial abnormalities were hyperplasia of epithelial cells with presence of mitotic cells (Fig. 1C), and rupture of pillar cells (Fig. 1D). The esophagus also presented hyperplasia of the epithelial cells (Fig. 2B) in all treatments, but at higher concentration this effect was more severe as denoted by the presence of cells in mitotic stage (Fig. 2C). The most relevant liver histopathology was dilatation of hepatic sinusoids (Fig. 3B), hepatocitomegaly and bi-nucleated hepatocytes (Fig. 3C), nuclear degeneration of hepatocytes and pyknotic nuclei (Fig. 3D). The histopathologies observed in the present study were more evident in concentrations of WSF higher than 50 % of petroleum, 16 % of diesel and 5% of gasoline.

Discussion

The concentrations of hydrocarbons present in the WSF of different crude oils found in the literature are variable. Neff et al. (2000) showed concentrations varying from 0.008 mg/L to 38.31 mg/L for different Australian crude oils. The analyses of 100% WSF of petroleum of the present investigation showed low concentration of BTEX, and high concentration of PAHs, and naphthalene corresponded to the highest portion of PAHs.

These results are in accordance with previous studies found in the literature (Anderson et al. 1974; Neff et al. 2000). Diesel and gasoline showed moderate and high concentrations of BTEX, respectively, and low concentrations of PAHs. Different studies demonstrated that BTEX represent a great part of hydrocarbons in WSF of diesel and gasoline (Anderson et al. 1974; Saeed and Al-Mutairi 1999; Neff et al. 2000).

Petroleum hydrocarbons are harmful to aquatic organisms, they can interfere on fish metabolism, causing metabolic stress, resulting in increased opercular movement and tail fin beating in Nile tilapia *Oreochromis niloticus* (Omoregie 2002), while in Australian bass *Macquaria novemaculeata*, petroleum induces metabolic stress, increasing oxygen consumption (Cohen and Nugegoda 2000). In the present investigation, water quality was hampered by WSF, chiefly decreasing the dissolved oxygen concentration and pH of the water. Omoregie and Ufodike (2000), also registered reduced levels of dissolved oxygen and pH when *O. niloticus* were exposed to WSF of petroleum, and it led to reduction of growth rate. However, the parameters of water quality observed here not interfered in the mortality of the larvae, according Boyd (1982).

Comparisons on toxicological effects of crude oil WSF are difficult, because hydrocarbon concentrations present in the petroleum are extremely variable according to its origin (Neff et al. 2000). Other factors can be described like the different methodologies applied for WSF preparation (Saeed and Al-Mutairi 1999; Singer et al. 2000), and distinct tolerance to crude oils presented by different species (Ramachandran et al. 2006). However, the toxicity of crude oil WSF seems to be higher in freshwater than in seawater species and this fact might be related to hydrocarbons solubility, and higher bioaccumulation in fish when salinity is reduced (Ramachandran et al. 2006;

Shukla et al. 2007). The LC_{50}-96 h of crude oil WSF for larvae of the freshwater Crimson-spotted rainbowfish *Melanotaenia fluviatilis* was around 40% (Pollino and Holdway 2002), while for Australian bass *M. novemaculeata* the LC_{50}-96 h of crude oil WSF was approximately 45% (Cohen and Nugegoda 2000). Higher and lower LC_{50}-96 h for marine species have been reported. Neff et al. (2000) working with marine silverside *Menidia beryllina* juveniles, estimated the LC_{50}-96h of three different crude oils WSF between 32 to 88%, whilst Saco-Álvarez et al. (2008) observed that the LC_{50}-96 h of Prestige oil WSF for *Cyprinodon variegates* varied from 49 to 53% of dilution. Finally, the LC_{50}-96h for larvae of *O. argentinensis* was equal to 70.7% of crude oil WSF. Considering the complications described above to establish comparisons between different ecotoxicological tests, this result should not lead to the conclusion that Brazilian petroleum is less toxic than others, or that larvae of *O. argentinensis* is more tolerant to hydrocarbons than other species.

Commercial diesel and gasoline were at least fivefold more toxic than Brazilian crude oil for marine pejerrey. Different investigations have shown that the WSF of refined petroleum products generally are more toxic than their respective crude oils (Anderson et al. 1974; Rayburn et al. 1996). Anderson et al. (1974) reported that number 2 fuel oil and bunker C WSF are more toxic than crude oil WSF. Comparable results were found between toxicity of WSF of diesel and gasoline for the mysid shrimp *Metamysidopsis insularis* (Mohammed 2005). On the other hand, Neff et al. (2000) found similar toxicity between WSF of diesel and Australian crude oil for juveniles of silverside *M. beryllina* and clownfish *Amphiprion clarkii*.

Barron et al. (1999) and Neff et al. (2000) evaluated the toxicity of WSF of different sources of petroleum hydrocarbons and concluded that the HPAs are not the major determinant of the toxicity of WSF. They pointed out that BTEX were the main responsible for the toxicity on marine fish and crustaceans, even considering they are very volatile. The higher toxicity of WSF of diesel and gasoline for *O. argentinensis* larvae, compared to the toxicity of WSF of petroleum confirms this idea, because the BTEX content was higher for the derivatives than for crude oil WSF. In addition, diesel and gasoline regularly contain additives, and these additives may have contributed to the toxicity of WSF, such as proposed by Neff et al. (2000).

The histology of gills and liver are good biomarkers to evaluate the toxicity of hydrocarbons (Brand et al. 2001). Gills are very sensitive and respond extremely fast to water pollution caused by petroleum and derivative fuels (Akaishi et al. 2004). Consequently, branchial morphology is used as a biomarker to the environmental contamination. In the present study, the WSF of Brazilian petroleum, diesel and gasoline induced hyperplasia of the epithelial cells of gill. Histopathologies are common in fish exposed to petroleum hydrocarbons. For example, Brand et al. (2001) working with juvenile pink salmon *Oncorhynchus gorbuscha* exposed to crude oil WSF observed the occurrence of hyperplasia on epithelium of secondary lamellae. It has also been reported hyperplasia with lamellar fusion on gills of juvenile Nile tilapia *Oreochromis niloticus* exposed to diesel WSF (Dede and Kaglo 2001). Khan (2003) also observed hyperplasia and hypertrophy of the lamellar epithelium in three species of marine flounder collected near an oil refinery, and related these alterations to the petroleum hydrocarbons.

According to Martinez et al. (2004), the rupture of pillar cells could generate an expressive lesion denominated telangiectasia lamellar as a response to xenobiotics exposition on branchial tissue. Telangiectasia was observed for juveniles of *O. gorbuscha* (Brand et al. 2001) exposed to crude oil WSF and for juveniles of *Prochilodus lineatus* exposed to diesel WSF (Simonato et al. 2008). However, the analyses conducted with larvae of *O. argentinensis* showed that WSF of petroleum, diesel and gasoline induced rupture of pillar cells in the gills, but it was not observed telangiectasia.

The esophagus also present hyperplasia of their epithelium. This histopathology was not previously described for petroleum hydrocarbons.

The presence of mitoses in the branchial and esophagus epithelia of pejerrey larvae are indicating the proliferation of this tissue in both organs, as previously described by Meissner and Diamandopoulos (1977). The observation of mitotic cells in the gills and esophagus suggest the induction of dysplasia in the current epithelia. In the present case, the mitotic cells observed in the gills and esophagus epithelia were attributed to the WSF of petroleum, diesel, and gasoline present in the seawater. The lesions observed on *O. argentinensis* larvae can be classified as moderate, and probably reactive to WSF, protecting the fish from exposition to hydrocarbons, as proposed by Simonato et al. (2008).

The pseudobranchs regulate the arterial blood flow to the ophthalmic artery (Takashima and Hibiya 1995) performing an important function of oxygenation of the fish eyes. The pseudobranchs also showed hyperplasia, indicating a possible negative effect on the vision of fish. This histopathology was not described previously in the literature.

The liver is the main organ of biotransformation and excretion of xenobiotics, and their presence it rapidly presents structural, biochemical and molecular alterations (Bernet et al. 1999). Marine pejerrey larvae exposed to WSF of petroleum, diesel and gasoline showed various histological alterations in the liver. Significant dilatation of hepatic sinusoids observed primarily in animals exposed to WSF of petroleum and diesel are probably indicating the increased blood volume received by liver to detoxify the organism. This alteration was not described before in the literature, at least for petroleum hydrocarbon toxicity in fish. The hepatocitomegaly observed in treatments of petroleum hydrocarbons is considered a hepatic histopathological biomarker for xenobiotics (Hinton 1994), indicating the functional activation of the liver in the presence of WSF of petroleum and derivatives fuels (Simonato et al. 2008). The presence of bi-nucleated and nuclear degeneration of hepatocytes, and piknotic nuclei are considered moderate lesions in the liver exposed to petroleum, diesel and gasoline WSF. Histopathological abnormalities in the liver of freshwater and saltwater species exposed to petroleum hydrocarbons were also described in the literature (Brand et al. 2001; Khan 2003; Akaishi et al. 2004). According to these authors, the most important damage caused by hydrocarbons was necrosis with cellular inflammatory response in the liver. Nevertheless, in *O. argentinensis* larvae, it was not observed severe lesions in the liver, such as necrosis.

The moderate histopathological alterations observed in the present investigation are probably associated to high growth rate presented by larvae during ontogeny. The short time of exposure to the toxicant might have also contributed, avoiding severe damages to the gills, esophagus, pseudobranchs, and liver.

In summary, the WSF of diesel and gasoline are more toxic than the Brazilian petroleum WSF for marine pejerrey *O. argentinensis* larvae. Further, all the toxicants induced a variety of histophatological alterations, principally on gills, pseudobranchs and liver of pejerrey larvae. Future studies are required in order to investigate the effects of sublethal concentrations of petroleum and derivative fuels on different life stages of *O. argentinensis*.

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Table 1 – Concentration (µg/L) of total monocyclic aromatic hydrocarbons (BTEX) and polycyclic aromatic hydrocarbons (PAHs) in the 100% water soluble fraction (WSF) of Brazilian petroleum, diesel and gasoline.

Hydrocarbon	Petroleum	Diesel	Gasoline
Benzene	17.86	289.06	nd
Toluene	nd	754.28	16,929.73
Ethylbenzene	nd	289.92	4,298.64
Xylene	425.28	1,771.82	14,822.03
Total BTEX	443.14	2,815.16	36,050.04
Naphthalene	26,186.00	31.23	403.04
Acenaphthelene	nd	nd	nd
Acenaphthene	nd	nd	nd
Fluorene	0.81	3.49	nd
Phenanthrene	2.62	3.89	nd
Anthracene	nd	nd	nd
Fluoranthene	nd	nd	nd
Pyrene	nd	nd	nd
Benz[a]anthracene	nd	nd	nd
Chrysene	nd	nd	nd
Benzo[b]fluoranthene	nd	nd	nd
Benzo[k]fluoranthene	nd	nd	nd
Benzo[a]pyrene	nd	nd	nd
Indeno[1.2.3-C.D]pyrene	nd	nd	nd
Dibenzo[a,h]anthracene	nd	nd	nd
Benzo[ghi]perylene	nd	nd	nd
Total HPAs	26,189.43	38.61	403.04
Σ HPAs and BTEX	26,632.57	2,853.77	36,453.08

*nd = not detected

Table 2. Accumulated mortality (%) of newly hatched larvae of marine pejerrey *Odontesthes argentinensis* exposed to water-soluble fraction (WSF) of petroleum, diesel and gasoline.

Time (h)	WSF Petroleum (%)					
	0	10	25	50	75	100
24	0	0	0	0	0	76.7
48	0	0	0	0	36.7	90.0
72	0	0	0	0	43.3	100
96	0	0	0	3.3	53.3	100
	WSF Diesel (%)					
	0	4	8	16	32	64
24	0	0	0	0	3.3	90.0
48	0	0	0	0	16.7	100
72	0	0	0	3.3	33.3	100
96	0	0	0	6.7	76.7	100
	WSF Gasoline (%)					
	0	1	2.5	5	10	20
24	0	0	0	0	90	100
48	0	0	3.3	30	100	100
72	0	0	6.7	33.3	100	100
96	0	0	6.7	40	100	100

Table 3. Median lethal concentration (LC_{50}-96h) and respective safe levels of water-soluble fraction of petroleum, diesel and gasoline for newly hatched larvae of marine pejerrey *Odontesthes argentinensis*.

Toxicant	LC_{50}-96h	Safe Level
Petroleum	70.68% (65.73 – 76.01)[a]	7.1%
Diesel	13.46 % (10.19 – 17.79)[b]	1.35%
Gasoline	5.48% (4.85 – 6.20)[b]	0.55%

Different letters at each line indicate statistical significant difference (P>0.05) after the Tukey Test.

Figure captions

Figure 1. Photomicrograph of pseudobranchs and gills of *Odontesthes argentinensis* larvae. (A) Control treatment showing normal structure of peudobranch (400 ×); (B) larvae exposed to 50% of petroleum water-soluble fraction **(**WSF) for 96 h, showing hyperplasia in the pseudobranch epithelium (400 ×); (C) larvae exposed to 50% of petroleum WSF for 96 h, showing mitotic cell of lamellar epithelium of gills (arrow; 1000 ×) (D) larvae exposed to 25% of petroleum WSF for 96 h, showing rupture of pillar cell in gills (arrow; 400 ×).

Figure 2. Photomicrograph of esophagus of *Odontesthes argentinensis* larvae. (A) Control treatment showing the structure of esophagus (200 ×); (B) larvae exposed to 5% of water-soluble fraction (WSF) of gasoline for 96 h, showing hyperplasia of epithelium (200 ×); (C) larvae exposed to 5% of gasoline WSF for 96 h, showing mitotic cells of lamellar epithelium (arrow; 1000 ×).

Figure 3. Photomicrograph of liver of *Odontesthes argentinensis* larvae. (A) Control treatment presenting normal structure of liver (400 ×); (B) larvae exposed to 32% of water-soluble fraction (WSF) of diesel for 96 h, showing dilatation of hepatic sinusoid (400 ×); (C) larvae exposed to 32% of diesel WSF for 96 h, showing hepatocitomegaly (arrow), and bi-nucleated hepatocytes (□; 1000 ×); (D) larvae exposed to 50% of petroleum WSF for 96 h, showing piknotic nuclei (arrow), and nuclear degeneration (□; 1000×).

Fig. 1

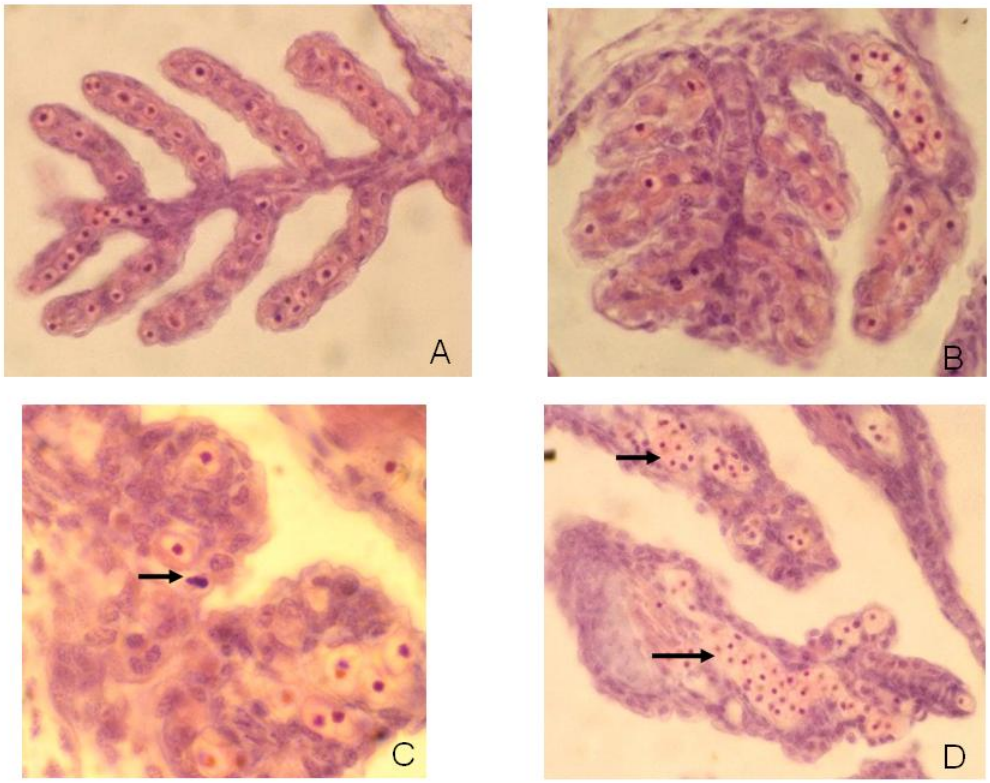


Fig. 2

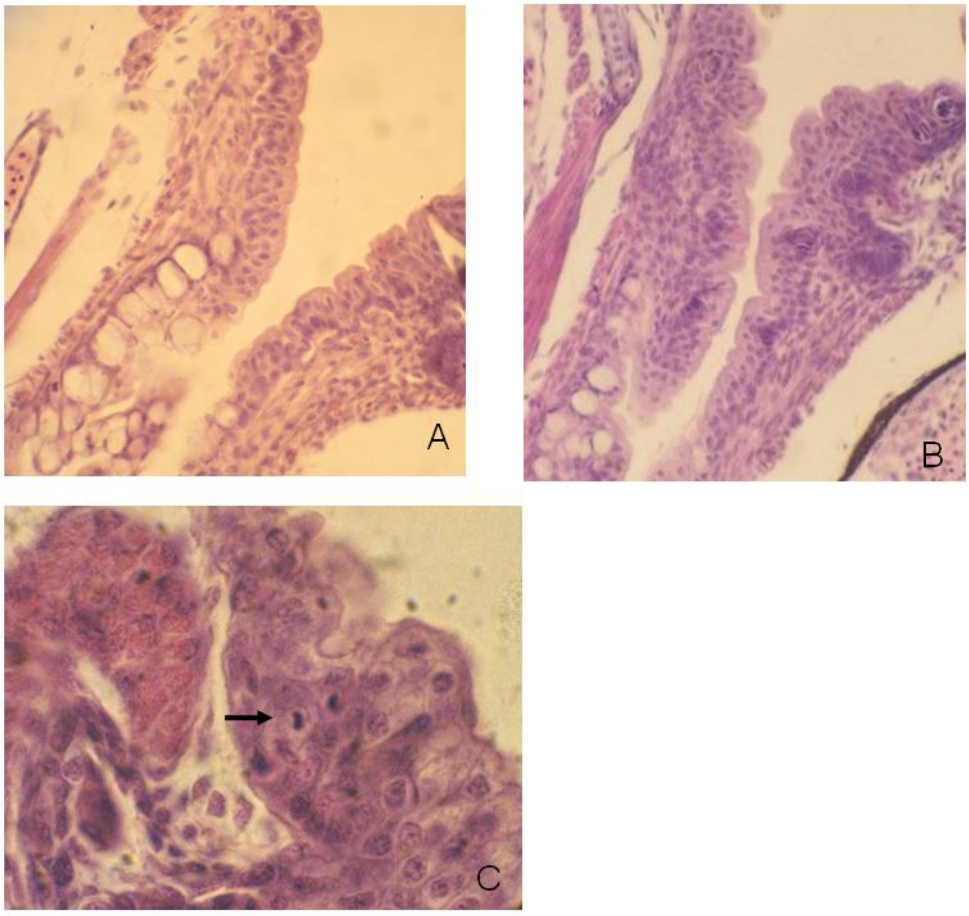
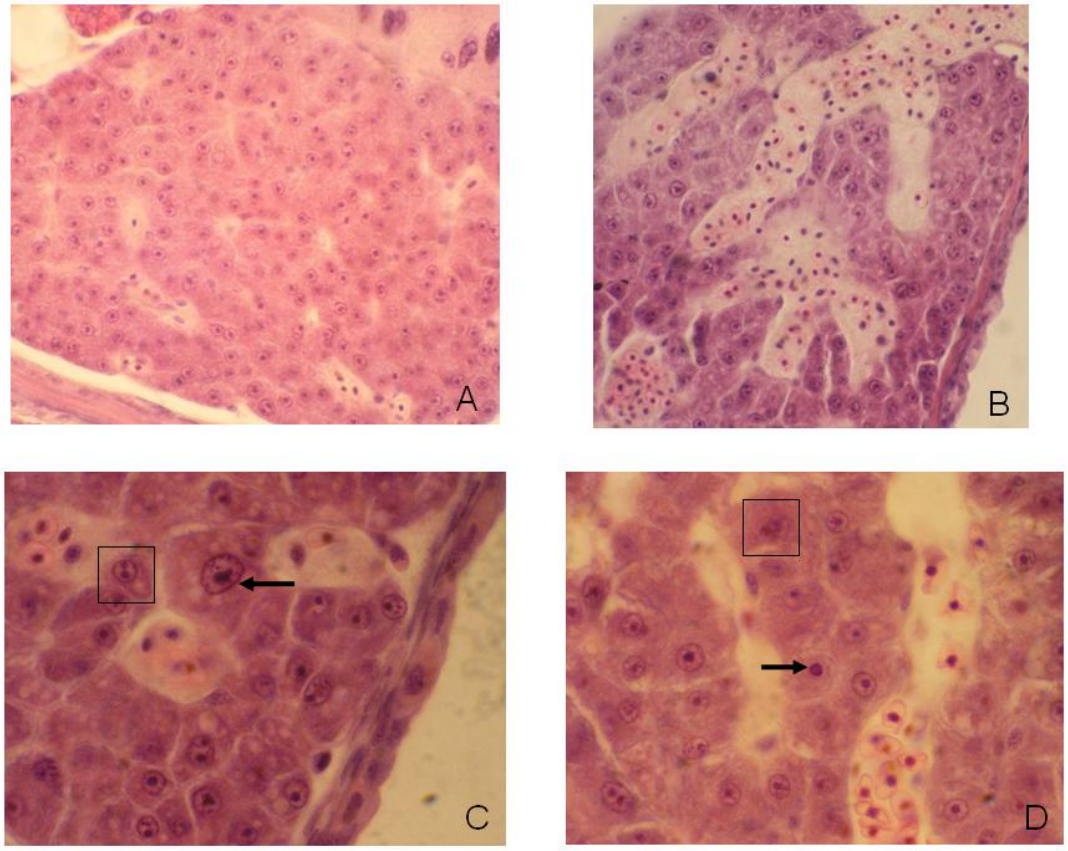


Fig. 3



5. CONCLUSÕES

O presente estudo demonstrou que os combustíveis diesel e a gasolina são pelo menos cinco vezes mais tóxicos que o petróleo brasileiro para larvas do peixe-rei marinho *O. argentinensis* expostas por 96 h durante ensaio de toxicidade aguda. No entanto, todos os poluentes induziram histopatologias moderadas e adaptativas nas brânquias, pseudobrânquias, esôfago e fígado das larvas do peixe-rei.