UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

EFEITOS HISTOPATOLÓGICOS DA EXPOSIÇÃO CRÔNICA DE LARVAS DO PEIXE-REI MARINHO *Odontesthes argentinensis* À FRAÇÃO SOLÚVEL DO PETRÓLEO EM ÁGUA (FSA)

EMELINE PEREIRA GUSMÃO

FURG

RIO GRANDE, RS

Março, 2009

Universidade Federal do Rio Grande
Programa de Pós-Graduação em Aqüicultura

EFEITOS HISTOPATOLÓGICOS DA EXPOSIÇÃO CRÔNICA DE LARVAS DO PEIXE-REI MARINHO *Odontesthes argentinensis* À FRAÇÃO SOLÚVEL DO PETRÓLEO EM ÁGUA (FSA)

EMELINE PEREIRA GUSMÃO

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.

Orientador: Prof. Dr. Kleber C. Miranda Filho

Rio Grande -RS- Brasil
Março, 2009

ÍNDICE

DEDICATÓRIA

Àqueles que, pelo dom de Deus, me deram a vida, e também me ensinaram a vivê-la:

Mara e Luiz.

AGRADECIMENTOS

Ao meu orientador Kleber Campos Miranda Filho, ou Klebinho, pelas horas de dedicação, pelos ensinamentos, pela paciência e pelo carinho, e também pelos bons conselhos que certamente ficarão para toda a vida.

Ao meu co-orientador, Luis André Sampaio, que sempre me auxiliou nos momentos difíceis, e na verdade sempre foi meu orientador também.

Ao Prof. Luis Romano, que, com sua experiência, cordialidade e disponibilidade, foi fundamental na finalização deste trabalho.

À Prof.ª Maria Isabel Machado, pela atenção maternal sempre dedicada aos bolsistas do PRH-27 da ANP, e ao Prof. Dr. Gilberto Griep, pelo auxílio e incentivo profissional através do PRH. Também aos colegas bolsistas do PRH, pela companhia.

Ao Renato Zacarias Silva, por todo o auxílio profissional e pela amizade, e pela companhia de trabalho no Laboratório de Morfologia Funcional. Também ao técnico Pedro Antonio Garcia, pelo auxílio no trabalho e companhia de chimarrão.

Ao colega e amigo Ricardo Rodrigues, grande companheiro de trabalho e de depois do trabalho também, excelente profissional e com quem sempre se pode contar.

Ao Cauê Moreira, sempre interessado, trabalhador e amigo em muitos momentos, e que, com sua simplicidade, consegue grandes feitos.

Aos amigos do PPGAq, colegas do mestrado e do laboratório de Piscicultura Marinha, que, além de colegas, tornaram-se grandes amigos e amigas, companheiros para todas as horas, boas ou difíceis. Pessoas batalhadoras, que me ensinaram grandes lições, e dos quais sentirei muita saudade, mas que estarão espalhados por esse país tornando-o melhor.

A todos os professores com os quais tive a oportunidade de conviver, ainda que alguns por pouco tempo, e que dedicaram seu tempo para passar adiante as suas experiências, acreditando em seus alunos.

À minha família, principalmente aos meus pais, Luiz e Mara, por todo o auxílio, força e amor que sempre me ofereceram sem esperar nada em troca.

Finalmente, aos meus amigos, que, ora perto, ora distantes, sempre me motivaram e me acompanharam nos momentos alegres e difíceis. Quem tem amigos como os meus, encontrou um grande tesouro.

1. RESUMO GERAL

Durante décadas o petróleo tem sido a principal fonte de energia para a humanidade e tem figurado como um dos principais poluentes encontrados em recursos aquáticos. Registros de derramamento de petróleo na região sul do Brasil são escassos, porém existem riscos devido à atividade de portos e indústrias relacionadas ao petróleo. O peixe-rei marinho *Odontesthes argentinensis* está distribuído desde São Paulo até o sul da Argentina, onde é um importante recurso pesqueiro e vem sendo considerado para aqüicultura. A toxicidade aguda e crônica da fração solúvel em água (FSA) do petróleo foi avaliada sobre o crescimento e efeitos histopatológicos em larvas de *O. argentinensis*. Larvas de 17 dias foram expostas às concentrações de 5%, 10%, 20%, 40%, 60% e 100% da FSA, somado ao grupo controle, por 96 h em experimento agudo. A concentração letal mediana (CL_{50}) foi estimada em 55% da FSA e o nível de segurança em 5,5% da FSA. O teste de toxicidade crônica foi conduzido com larvas recém-eclodidas foram expostas a concentrações subletais (2,5%, 5%, 10% e 20% da FSA), mais o controle sem adição da FSA de petróleo (todos com três repetições) em sistema semi-estático por 21 dias. A sobrevivência e o crescimento das larvas expostas a 20% da FSA foram significativamente menores que as outras concentrações. Alterações histopatológicas foram encontradas nas brânquias (hemorragia, telangiectasia lamelar, necrose, hipertrofia e hiperplasia), no fígado (basofilia, hipertrofia, cariorrese, cariopicnose) e nos rins (alterações nucleares e alargamento dos glomérulos). As larvas de peixe-rei podem sofrer danos que indicam a severidade de uma possível exposição à FSA do petróleo. O biomarcador histopatológico se mostrou bastante sensível em relação à exposição aguda e crônica de *O. argentinensis* ao petróleo.

2. ABSTRACT GERAL

The toxicological analysis of petroleum is usually performed on the basis of water-soluble fraction (WSF). The WSF contains a mixture of polycyclic aromatic hydrocarbons (PAHs), volatile hydrocarbons, phenols and heterocyclic compounds, considered deleterious to aquatic biota. Marine "pejerrey" *Odontesthes argentinensis* (Teleostei:Atherinopsidae) occurs from São Paulo, Brazil, to the South of Argentina, and it has a great commercial importance in local fisheries and a high potential for aquaculture. The aim of this work was to evaluate the histopathological effects in "pejerrey" larvae exposed to petroleum WSF. For the petroleum WSF, the total PAHs estimated by gas chromatography (GC) with a mass spectrometer (MS) was 197.83 μg L^{-1}, and 106.11 μg L^{-1} for total BTEX, estimated by GC with a flame ionization detector (FID) and a headspace Turbomatrix. For the acute toxicity test, 17 days old larvae were exposed to 5%, 10%, 20%, 40%, 60% and 100% of WSF, plus one control. After 96 hours of experiment, the LC_{50} (55% of WSF) and the safe level (5.5% of WSF) were estimated for the species. The chronic toxicity test was conducted with newly hatched larvae exposed to sublethal concentrations of WSF (2.5%, 5%, 10% and 20% of WSF), plus one control, in semi-static system for 21 days. Survival and growth were affected by petroleum WSF, and were significantly lower in the highest concentration. Several histopathological changes were found in the chronic toxicity test, which shows that histopathological biomarker was very sensitive in *O. argentinensis* larvae. The application factor used to estimate the safe level for toxic exposure to petroleum must be used with attention for "pejerrey" larvae.

3. INTRODUÇÃO GERAL

As zonas costeiras são definidas como faixas de transição entre o continente e o oceano, apresentando dinâmicas naturais bastante intensas (Clark, 2001). Ao longo da evolução, a humanidade sempre apresentou tendências a se distribuir em regiões costeiras (Kennish, 1997). A densidade demográfica nessas áreas é tende a ser maior que em regiões continentais interiores. Como conseqüência constata-se uma maior ocorrência de compostos xenobióticos passíveis de gerar efeitos deletérios à biota aquática (Waldichuk, 1989; Tanabe e Tatsukawa, 1992; Kennish, 1997).

Dentre os principais poluentes que afetam os ambientes aquáticos, destacam-se os metais pesados, compostos orgânicos sintéticos, hidrocarbonetos de petróleo e esgotos urbanos e industriais (Baird, 2002). Entre estes, os efeitos tóxicos do petróleo são bem reconhecidos, devido à sua ampla utilização pela humanidade (Clark, 2001).

O petróleo tem sido utilizado desde as civilizações antigas, entretanto, nas últimas décadas, é empregado exaustivamente como combustíveis, solventes, plásticos, entre outros. Diversas atividades da indústria do petróleo, desde a extração até o consumo de seus derivados, podem ser danosas ao ambiente, causando desde a poluição atmosférica, através do refino e da combustão do petróleo, até a contaminação do meio aquático, através de efluentes e derramamentos de petróleo. Com o aumento da produção marinha de petróleo aliado à transferência e estocagem em zonas costeiras, os níveis de contaminação das águas principalmente por hidrocarbonetos do petróleo também se elevaram. Alguns exemplos conhecidos de derramamentos de petróleo são, o do navio *Prestige*, na costa espanhola em 2002, derramando cerca de 63 mil toneladas

de petróleo na água, e o do navio *Exxon Valdez*, no Alasca (EUA), em 1989, derramando 37 mil toneladas de petróleo. Porém, os maiores acidentes documentados lançaram quase 300 toneladas de petróleo no ambiente aquático (ITOPF, 2007).

O petróleo e o gás natural são responsáveis por parcela significativa da matriz energética brasileira e deverão permanecer com demanda crescente nos próximos anos, sendo que 80% do petróleo nacional são produzidos através de plataformas marítimas localizadas ao longo da costa brasileira (CONAMA, 2007).

A Resolução do CONAMA nº 357, de 17 de março de 2005, dispõe sobre a classificação de corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamentos de efluentes. A Resolução do CONAMA nº 393, de 15 de agosto de 2007, dispõe sobre o descarte contínuo de água de processo ou de produção em plataformas marítimas de petróleo e gás natural. De acordo com estas resoluções, as águas marinhas depois de utilizadas, devem atender a padrões de descarte de óleos e graxas, e ser objeto de estudos de monitoramento, nos quais ensaios ecotoxicológicos são procedimentos necessários para o monitoramento da água marinha (incluindo a água produzida), na qual não deve ser verificado efeito tóxico crônico a organismos. A qualidade dos ambientes aquáticos poderá ser avaliada por indicadores biológicos, quando apropriado, utilizando-se organismos e/ou comunidades aquáticas, o que torna cada vez mais necessária a realização de estudos ecotoxicológicos e a ampliação do conhecimento sobre a biota aquática marinha brasileira.

Ziolli e Jardim (2002) afirmam que, após um derramamento de petróleo, a camada superficial de óleo é retirada tanto por métodos de remoção mecânica quanto de absorção. Em termos de efeitos ambientais e ecológicos da poluição com petróleo, o

grau em que os componentes se dissolvem na água marinha antes da remoção da camada oleosa é muito importante. A fração dissolvida é prontamente ingerida por organismos da base da cadeia alimentar, assim sendo concentrada e acumulada em organismos de níveis tróficos mais elevados.

A fração solúvel em água (FSA) do petróleo é a fase aquosa orgânica enriquecida em contato com o óleo derramado (Ziolli e Jardim, 2002). Esse enriquecimento é causado pela dissolução de componentes de relativamente baixo peso molecular presentes em óleo bruto, principalmente os hidrocarbonetos aromáticos, fenóis e compostos heterocíclicos contendo nitrogênio e enxofre (Akaishi et al, 2004).

O petróleo é constituído por uma gama de hidrocarbonetos na qual destacam-se, pela elevada toxicidade, os hidrocarbonetos policíclicos aromáticos (HPAs). Ainda que sejam encontrados naturalmente, atividades antrópicas têm aumentado consideravelmente os níveis ambientais de HPAs (Hylland, 2006). São encontradas quatro categorias de HPAs no ambiente marinho: (1) biogênicos, ou produzidos por organismos, (2) pirogênicos, ou derivados de processos de incineração, (3) petrogênicos, ou derivados de combustíveis fósseis, e (4) diagênicos, ou derivados de processos de transformação de solos e sedimentos. Somente os HPAs pirogênicos e petrogênicos possuem importância quantitativa nos ecossistemas marinhos. Introduções diretas incluem efluentes domésticos, escoamentos de estradas e derramamentos de óleo (Hylland, 2006).

Segundo a US EPA (U.S. Environmental Protection Agency), há 16 HPAs prioritários, entre os milhares existentes na natureza: naftaleno, acenaftileno, acenafteno, fluoreno, fenantreno, antraceno, fluoranteno, pireno, benzo(a)antraceno, criseno, benzo(b)fluoranteno, benzo(k)fluoranteno, benzo(a)pireno, indeno(1,2,3-

cd)pireno, dibenzo(a,h)antraceno e benzo(ghi)perileno; dentre estes, diversos já tiveram efeitos mutagênicos e carcinogênicos comprovados. Os HPAs mais leves (2-3 anéis benzênicos), como o naftaleno, acenafteno e fluoranteno, são mais hidrossolúveis, tornando-se mais biodisponíveis e causando uma maior toxicidade aguda nos organismos. Já os HPAs mais pesados (4-10 anéis benzênicos), como o pireno, benzo[a]pireno e benzo[a]antraceno, são menos hidrossolúveis e menos disponíveis, porém causam uma maior toxicidade crônica nas biota, por serem mais lipossolúveis.

Uma parte dos hidrocarbonetos monoaromáticos do petróleo é representada pelos compostos benzeno, tolueno, etilbenzeno e xileno (BTEX), sendo estes compostos altamente hidrossolúveis e rapidamente absorvidos pela biota (Stephens *et al.*, 1997). Porém, são poucos os estudos que levam em consideração os BTEX. Tal fato decorre principalmente da sua elevada volatilidade no ambiente e da dificuldade na análise quantitativa dos mesmos (Stephens et al., 1997; Dórea et al., 2007).

De acordo com Kennish (1997), o petróleo pode causar diversos impactos sobre a biota, que podem persistir por longos períodos de tempo:

- danos físicos (redução da luminosidade, impedimento de trocas com o ambiente);
- danos químicos (alteração do pH e da temperatura, redução do oxigênio disponível);
- danos biológicos (redução do alimento disponível, patologias).

A toxicologia pode ser conceituada como sendo o estudo dos efeitos adversos causados por compostos químicos sobre os seres humanos e ela possui uma rica história através da evolução da humanidade. Já a ecotoxicologia pode ser considerada um novo

ramo da toxicologia e é focada na saúde da flora e da fauna e suas populações, comunidades e ecossistemas. Há uma biocomplexidade nesse tipo de estudo, pois ainda há várias lacunas a serem preenchidas sobre um grande número de efeitos e espécies. Para lidar com efeitos em vários níveis da organização biológica, ainda é necessário extrapolar resultados entre as espécies (Di Giulio e Hinton, 2008).

Os testes de toxicidade aguda e crônica são amplamente utilizados na ecotoxicologia aquática, seguindo padrões e metodologias já estabelecidos por órgãos normativos nacionais e internacionais, como a Associação Brasileira de Normas Técnicas (ABNT), USEPA, European Environment Agency (EEA), entre outros. A toxicidade aguda é definida como efeito de curto prazo, após uma rápida exposição a elevadas concentrações de um contaminante, e a toxicidade crônica é definida como efeitos em longo prazo, geralmente após exposições contínuas e subletais ao contaminante (NRC, 2003). Tais testes podem ser importantes em avaliações de risco ecológico e atividades de biomonitoramento ambiental (Di Giulio e Hinton, 2008). De acordo com Claisse (1989), o uso de organismos como bioindicadores e biomonitores de poluição vem sendo aplicado em atividades de monitoramento da contaminação ambiental desde a década de 80.

Uma das formas de se constatar o efeito tóxico gerado pela exposição a um contaminante se dá através do estudo com biomarcadores. Segundo Monserrat *et al.* (2006), os biomarcadores são medidas de fluidos corporais, células, tecidos ou até mesmo parâmetros comportamentais que indicam em termos bioquímicos ou celulares a presença de contaminantes, sendo assim são tidos como importantes ferramentas de avaliação de contaminação em ambientes aquáticos.

O uso da histopatologia traz diversas vantagens para estudos de monitoramento ambiental. Comparando com os testes toxicológicos clássicos, a sensitividade do monitoramento histológico é melhorada, visto que os efeitos no nível histológico serão visíveis em doses mais baixas que no nível de mortalidade ou mudanças comportamentais, o que contribui para o bem-estar animal (Wester *et al.*, 2002). Outra vantagem é poder trabalhar com peixes pequenos, ovos, larvas e juvenis, e a possibilidade de uma visualização rápida de vários órgãos relevantes ao mesmo tempo.

Diversos estudos têm sido conduzidos utilizando a histopatologia como biomarcador, inclusive para estudos toxicológicos com petróleo e combustíveis derivados (Myers *et al.* 1998; Grinwis *et al.* 2000; Simpson *et al.* 2000; Nero *et al.* 2006a,b). Alterações histopatológicas podem ser o resultado de mudanças bioquímicas e fisiológicas adversas em um organismo. Assim sendo, a histopatologia pode indicar rapidamente órgãos-alvo, tecidos, células e organelas que estejam afetadas e, em muitos casos, é possível distinguir lesões induzidas por tóxicos daquelas lesões provenientes de doenças infecciosas (Hinton e Laurén, 1990).

De acordo com Bernet *et al.* (1999), dentre os órgãos indicados para o estudo histopatológico em peixes, destacam-se principalmente as brânquias, expostas permanentemente a potenciais agentes tóxicos, o fígado e os rins, os quais possuem papéis essenciais no metabolismo, excreção e osmorregulação.

As brânquias realizam uma variedade de funções fisiológicas críticas incluindo a troca de gases, ionorregulação, manutenção do balanço ácido-base e excreção de compostos nitrogenados. Conforme descrito anteriormente, este órgão está continuamente exposto a contaminantes e poluentes presentes no meio externo (Hinton e Laurén, 1990).

O fígado é importante em muitos aspectos da nutrição, incluindo armazenamento de lipídios e carboidratos. É também sítio principal do sistema metabolizante de tóxicos mediado pelo sistema citocromo P-450, onde se encontram enzimas e co-fatores relacionados com a fase-I (redução e oxidação) e com a fase-II (conjugação) da biotransformação de xenobióticos (Hinton e Laurén, 1990). Os peixes apresentam grande eficiência nas duas fases, podendo, por exemplo, metabolizar rapidamente HPAs, não ocorrendo bioacumulação desses compostos (Hylland, 2006). Quando estes compostos são conjugados para outros mais hidrofílicos para excreção, tais metabólitos podem também apresentar alta toxicidade (Hinton e Laurén, 1990).

O rim dos peixes recebe grande parte do sangue pós-branquial, e lesões renais podem ser bons indicadores da poluição ambiental. Porém, ainda há poucos estudos utilizando alterações renais patológicas como indicadores de toxicidade ou poluição ambiental (Hinton e Laurén, 1990; Silva e Martinez, 2007).

Mesmo que os compostos mais tóxicos da FSA do petróleo sejam voláteis, organismos aquáticos, como os peixes, podem absorver rapidamente parte dessas substâncias, com conseqüências graves para a organização biológica. Estudos têm sido desenvolvidos avaliando a ecotoxicidade da FSA em peixes (Lockhart et al., 1996; Omoregie & Ufodike, 2000; Dede & Kaglo, 2001; Akaishi et al., 2004) e têm demonstrado que os peixes são ótimos bioindicadores da contaminação de HPAs em amostras marinhas (Gagnon & Holdway, 1999; Martínez-Gómez et al., 2006; Morales-Caselles et al., 2006; Ramachandran et al., 2006). Em embriões, ovos e larvas de peixes marinhos, comprovou-se que a FSA do petróleo tem efeito teratogênico e mutagênico, além de outras anormalidades durante o desenvolvimento, como patologias nas brânquias e no fígado (Brand et al., 2001; Middaugh et al., 2002; Pollino e Holdway,

2002). Já foram relatados também efeitos deletérios da FSA do petróleo sobre o crescimento e metabolismo de peixes (Woodward et al., 2001; Khan, 2003; Davoodi e Claireaux, 2007).

Quando um critério específico de classificação de lesões é aplicado uniformemente, a variação entre investigadores pode ser reduzida (Wester *et al.*, 2002). Segundo Bernet *et al.* (1999), a importância de técnicas e avaliações padronizadas baseia-se na possibilidade da quantificação das lesões, de avaliação estatística e também da comparação entre diferentes estudos. A comparação entre índices de lesões pode ser mais fácil que a comparação entre descrições morfológicas ou patológicas.

Os peixes são amplamente utilizados em estudos de toxicologia aquática. A escolha da espécie depende de exigências regulatórias (órgãos ambientais), representatividade, hábitos locais, disponibilidade, conhecimento e experiência com a espécie. Algumas espécies já utilizadas na ecotoxicologia aquática podem ser facilmente obtidas de fontes de aqüicultura, como a truta arco-íris (*Oncorhynchus mykiss*) e a carpa comum (*Cyprinus carpio*) (Wester *et al.*, 2002).

A aqüicultura é uma atividade conhecida por proporcionar uma importante fonte de proteína animal em diversas partes do mundo (e.g. produção de 47 milhões de toneladas em 2006) (FAO, 2006), podendo ser também uma fonte importante de organismos aquáticos para a realização de estudos ecotoxicológicos.

Nos últimos anos, algumas espécies de peixe-rei têm sido utilizadas em estudos ecotoxicológicos, como *Menidia beryllina* (Teleostei: Atherinopsidae), já aprovada pela EPA como espécie marinha padronizada para testes toxicológicos agudos e crônicos (Anderson et al., 1974; Hemmer et al., 1992; Al-Yakoob et al., 1996). O peixe-rei *Odontesthes argentinensis* (Valenciennes, 1835) (Teleostei: Atherinopsidae) encontra-

se distribuído desde São Paulo até o sul da Argentina (Brian & Dyer, 2006), tendo grande importância nas pescarias da região. Esta espécie está adaptada tanto ao estuário (e.g. estuário da Lagoa dos Patos - RS) quanto à região marinha costeira, formando duas populações distintas. A desova da população estuarina ocorre em enseadas rasas entre agosto e setembro, e no mar entre agosto e dezembro (Moresco e Bemvenuti, 2006). Os ovos podem ser encontrados na praia em períodos de ressaca. As larvas, quando eclodem, possuem vitelo e são relativamente grandes (entre 6 a 8 mm) quando comparadas a outras espécies de peixes marinhos, e eclodem com a boca e o ânus abertos, podendo receber alimentação exógena.

Os estuários e baías semi-fechadas são sistemas caracterizados pela baixa taxa de troca relativa ao seu volume, sendo extremamente suscetíveis ao lançamento de contaminantes, dentre os quais se destacam os HPAs (Kennish, 1997, Medeiros et al., 2005). Apesar de caracterizar-se como um ambiente de baixa diversidade, por causa da alta instabilidade ambiental, diversas espécies depende dos estuários (Seeliger et al., 1997), o que aumenta a necessidade de avaliação do impacto causado por contaminantes através da utilização de espécies bioindicadores representativas da região.

Vários estudos têm sido realizados no sul do Brasil com *O. argentinensis* visando sua introdução nas atividades de aqüicultura (Tesser e Sampaio, 2001; Sampaio, 2006), incluindo testes de toxicidade envolvendo parâmetros físico-químicos como:

- temperatura (Sampaio & Pissetti, 2001);
- salinidade (Phonlor & Sampaio, 1992);
- compostos nitrogenados (Sampaio & Minillo, 2000; Sampaio et al., 2006).

Há um grande potencial de utilização da espécie tanto para introdução na aqüicultura, quanto para a realização de ensaios ecotoxicológicos visando o futuro biomonitoramento da região costeira onde a espécie encontra-se distribuída.

Há poucos estudos sobre os impactos do petróleo brasileiro, geralmente caracterizado como um petróleo pesado, sobre organismos marinhos no Brasil. Porém, é importante separar os efeitos de um derrame de outros fatores que afetem as atividades de aqüicultura e pesca, para determinar o real impacto do contaminante sobre a biota (ITOPF, 2007). Os organismos presentes no local de um derramamento e seu estágio de desenvolvimento precisam ser levados em consideração antes que uma ação remediadora possa ser tomada (Carls et al., 1999).

Ainda que não haja registros de derramamentos de petróleo de médio e grande porte na região costeira do sul do Brasil, os riscos existem em função das atividades de navegação e à presença de indústrias vinculadas ao petróleo e seus derivados, que trabalham com refino, estocagem e transporte. Assim, conforme descrito anteriormente, como o peixe-rei é uma espécie de interesse comercial e uma candidata para aqüicultura, utilizando a região para reprodução e crescimento, é necessário avaliar os possíveis efeitos agudos e crônicos de um derramamento de óleo na região e também a possibilidade de utilizar a espécie como um bioindicador ou biomonitor de contaminação do estuário e da região costeira adjacente.

4. REFERÊNCIAS BIBLIOGRÁFICAS

Akaishi, F.M., Assis, H.C.S., Jakobi, S.C.G., Eiras-Stofella, D.R., St-Jean, S.D., Courtenay, S.C., Lima, E.F., Wagener, A.L.R., Scofield, A.L., Ribeiro, C.A.O.,

2004. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax* sp.) after Waterborne and acute exposure to water soluble fraction (WSF) of crude oil. Arch. Environ. Contam. Toxicol. 46, 244-253.

Al-Yakoob, S.N., Gundersen, D., Curtis, L., 1996. Effects of the water-soluble fraction of partially combusted crude oil from Kuwait's oil fires (from desert storm) on survival and growth of the marine fish *Menidia beryllina*. Ecotoxicol. Environ. Saf. 35, 142-149.

Anderson, J.W., Neff, J.M., Cox, B.A., Tatem, H.E., Hightower, G.M., 1974. Characteristics of dispersions and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. Mar. Biol. 27, 75-88.

Baird, C., 2002. Química ambiental. Bookman, Porto Alegre.

Bernet, D., Schmidt, H., Meier, W., Burckhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis. 22, 25-34.

Brand, D.G., Fink, R., Bengeyfield, W., Birtwell, I.K., Mcallister, C.D., 2001. Salt-water acclimated pink salmon fry (*Oncorhynchus gorbusha*) develop stress-related visceral lesions after 10-day exposure to sublethal concentrations of the water-soluble fraction of North Slope crude oil. Toxicol. Pathol. 29 (5), 574-584.

Brian, S., Dyer, H., 2006. Systematic revision of the South American silversides (Teleostei, Atheriniformes). Biocell 30 (1), 69-88.

Carls, M.G., Rice, S.D., Hose, J.E., 1999. Sensitivity of fish embryos to weathered crude oil: Part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval Pacific herring (*Clupea pallasi*). Environ. Toxicol. Chem. 18 (3), 481-493.

Claisse, D., 1989. Chemical contamination of French coasts: The results of a ten years Mussel Watch. Mar. Pollut. Bull. 20, 523-528.

Clark, R., 2001. Marine pollution. Oxford University Press, Oxford.

CONAMA (Conselho Nacional do Meio Ambiente), 2005. Resolução n° 357 de 17 de março de 2005.

CONAMA (Conselho Nacional do Meio Ambiente), 2007. Resolução n° 393 de 08 de agosto de 2007.

Davoodi, F., Claireaux, G., 2007. Effects of exposure to petroleum hydrocarbons upon the metabolism of the common sole *Solea solea*. Mar. Pol. Bul. 54, 928-934.

Dede, E.B., Kaglo, H.D., 2001. Aqua-toxicological effects of water soluble fractions (WSF) of diesel fuel on *O. niloticus* fingerlings. J. Appl. Sci. Environ. Manag. 5 (1), 93-96.

Di Giulio, R.T., Hinton, D.E., 2008. Toxicology of fishes. CRC Press, USA.

Dórea, H.S., Bispo, J.R.L., Aragão, K.A.S., Cunha, B.B., Navickiene, S., Alves, J.P.H., Romão, L.P.C., Garcia, C.A.B., 2007. Analysis of BTEX, PAHs and metals in the oilfield produced water in the State of Sergipe, Brazil. Microchem. J. 85, 234-238.

FAO – Food and Agriculture Organization of the United Nations, 2006. The state of world fisheries and aquaculture - 2006. Rome, Italy.

Gagnon, M.M., Holdway, D.A., 1999. Metabolic enzyme activities in fish gills as biomarkers of exposure to petroleum hydrocarbons. Ecotoxicol. Environ. Saf. 44, 92-99.

Grinwis, G., Besselink, H., Van Den Brandhof, E., Bulder, A., Engelsma, M., Kuiper, R., Wester, P., Vaal, M., Vethaak, A., Vos, J., 2000. Toxicity of TCDD in European flounder (*Platichthys flesus*) with emphasis on histopathology and

cytochrome P450 1A induction on several organ systems. Aquat. Toxicol. 50, 387-401.

Hemmer, M., Middaugh, D., Comparetta, V., 1992. Comparative acute sensitivity of larval topsmelt, Atherinops affinis, and inland silverside, *Menidia beryllina*, to 11 chemicals. Environ. Toxicol. Chem. 11, 401-408.

Hinton, D., Laurén, D., 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. Am. Fish. Soc. Symp. 8, 51-66.

Hylland, K., 2006. Polyciclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems. J. Toxicol. Environ. Health, Part A 69, 109-123.

ITOPF – The International Tanker Owners Pollution Federation Limited, 2007. ITOPF Handbook 2007/2008. Available at www.itopf.com.

Khan, R.A., 2003. Health of flatfish from localities in Placentia Bay, Newfoundland, contaminated with petroleum and PCBs. Arch. Environ. Contam. Toxicol. 44, 485-492.

Kennish, M., 1997. Practical handbook of estuarine and marine pollution. CRC Press, USA.

Lockhart, W.L., Duncan, D.A., Billeck, B.N., Dannell, R.A., Ryan, M.J., 1996. Chronic toxicity of the "water soluble fraction" of Norman Wells crude oil to juvenile fish. Spill Sci. Technol. Bull. 3 (4), 259-262.

Martínez-Gómez, C., Campillo, J.A., Benedicto, J., Fernández, B., Valdés, J., García, I., Sanchez, F., 2006. Monitoring biomarkers in fish (*Lepidorhombus boscii* and *Callionymus lyra*) from the northern Iberian shelf after the Prestige oil spill. Mar. Pollut. Bull. 53, 305-314.

Medeiros, P.M., Bicego, M.C., Castelao, R.M., Del Rosso, C., Fillmann, G., Zamboni, A.J., 2005. Natural and anthropogenic hydrocarbon inputs to sediments of Patos Lagoon Estuary, Brazil. Environ. Int. 31, 77-87.

Middaugh, D.P., Chapman, P.J., Shelton, M.E., Mckenney Jr, C.L., Courtney, L.A., 2002. Effects of fractions from biodegraded Alaska North Slope Crude Oil on embryonic inland silversides, *Menidia beryllina*. Arch. Environ. Contam. Toxicol. 42, 236-243.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2006. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comp. Biochem. Physiol., Part C: In press.

Morales-Caselles, C., Jiménez-Tenorio, N., Canales, M.L.G., Sarasquete, C., Delvales, T.A., 2006. Ecotoxicity of sediments contaminated by the oil spill associated with the tanker "Prestige" using juveniles of the fish *Sparus aurata*. Arch. Environ. Contam. Toxicol. 51, 652-660.

Moresco, A., Bemvenuti, M.A., 2006. Biologia reprodutiva do peixe-rei *Odontesthes argentinensis* (Valenciennes) (Atherinopsidae) da região marinha costeira do sul do Brasil. Rev. Bras. Zool. 23 (4), 1168-1174.

Myers, M.S., Johnson, L.L., Hom, T., Collier, T.K., Stein, J.E., Varanasi, U., 1998. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: relationships with other biomarkers of contaminant exposure. Mar. Environ. Res. 45(1), 47-67.

Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., Dixon, D.G., 2006a. The effects of salinity on naphthenic acid toxicity to yellow perch: gill and liver histopathology. Ecotoxicol. Environ. Saf. 65, 252-264.

Nero, V., Farwell, A., Lister, A., Van Der Kraak, G., Lee, L.E.J., Van Meer, T., Mackinnon, M.D., Dixon, D.G., 2006b. Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Cassius auratus*) exposed to oil sands process-affected water. Ecotoxicol. Environ. Saf. 63, 365-377.

NRC, 2003. Oil in the sea III: Inputs, fates, and effects. Committee on Oil in the Sea: Inputs, Fates, and Effects. National Research Council.

Omoregie, E., Ufodike, B.C., 2000. Effects of water soluble fractions of crude oil on growth of the Nile tilapia, *Oreochromis niloticus* (L.). Bull. Environ. Contam. Toxicol. 64, 601-605.

Phonlor, G., Sampaio, L.A., 1992. Effects of salinity on growth and survival of *Odontesthes argentinensis* larvae. Arq. Biol. e Tecnol. 35, 153-155.

Pollino, C.A., Holdway, D.A., 2002. Toxicity testing of crude oil and related compounds using early life stages of the crimson-spotted rainbowfish (*Melanotaenia fluviatilis*). Ecotox. Environ. Saf. 52, 180-189.

Ramachandran, S.D., Sweezey, M.J., Hodson, P.V., Boudreau, M., Courtenay, S.C., Lee, K., King, T., Dixon, J.A., 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Mar. Pollut. Bull. 52, 1182-1189.

Sampaio, L.A., 2006. Production of "pejerrey" *Odontesthes argentinensis* fingerlings: A review of current techniques. Biocell 30 (1), 121-123.

Sampaio, L.A., Minillo, A., 2000. Viabilidade do uso de larvas do peixe-rei *Odontesthes argentinensis* em testes de toxicidade: efeitos da salinidade e da

temperatura sobre a toxicidade aguda da amônia. In: Ecotoxicologia, perspectivas para o século XXI, São Carlos: RiMa, p. 545-553, 2000.

Sampaio, L.A., Pissetti, T.L., 2001. Efeito da temperatura sobre o desenvolvimento embrionário e larval do peixe-rei marinho *Odontesthes argentinensis*. In: XIV Semana Nacional de Oceanografia, Rio Grande.

Sampaio, L.A., Pissetti, T.L., Morena, M., 2006. Toxicidade aguda do nitrito em larvas do peixe-rei marinho *Odontesthes argentinensis* (Teleostei, Atherinopsidae). Ciênc. Rural 36 (3), 1008-1010.

Seeliger, U., Odebrecht, C., Castello, J.P., 1997. Subtropical convergence environments: the coast and the sea in the Southern Atlantic. Springer-Verlag, Germany.

Silva, A.G., Martinez, C.B.R., 2007. Morphological changes in the kidney of a fish living in an urban stream. Environ. Toxicol. Pharmacol. 23, 185-192.

Simpson, M.G., Parry, M., Kleinkauf, A., Swarbreck, D., Walker, P., Leah, R.T., 2000. Pathology of the liver, kidney and gonad of flounder (*Platichthys flesus*) from a UK estuary impacted by endochrine disrupting chemicals. Mar. Environ. Res. 50, 283-287.

Stephens, S.M., Alkindi, A.Y.A., Waring, C.P., Brown, J.A., 1997. Corticosteroid and thyroid responses of larval and juvenile turbot exposed to the water-soluble fraction of crude oil. J. Fish Biol. 50, 953-964.

Tanabe, S., Tatsukawa, R., 1992. Chemical modernization and vulnerability of cetaceans: increasing toxic threat of organochlorine contaminants. In: Persistent pollutants in marine ecosystems. Oxford: Pergamon Press, p. 161-177.

Tesser, M., Sampaio, L.A., 2001. Growth of pejerrey larvae (*Odontesthes argentinensis*) on different prey densities. Atlântica 23, 153-159.

Waldichuk, M., 1989. The state of pollution in the marine environment. Mar. Pollut. Bull. 20, 598-602.

Wester, P.W., Van Der Ven, L.T.M., Vethaak, A.D., Grinwis, G.C.M., Vos, J.G., 2002. Aquatic toxicology: opportunities for enhancement through histopathology. Environ. Toxicol. Pharmacol. 11, 289-295.

Woodward, D.F., Mehrle Jr., P.M., Mauck, W.L., 1981. Accumulation and sublethal effects of a Wyoming crude oil in cutthroat trout. Trans. Am. Fish. Soc. 110, 437-445.

Ziolli, R.L., Jardim, W.F., 2002. Operational problems related to the preparation of the seawater soluble fraction of crude oil. J. Environ. Monit. 4, 138-141.

5. ARTIGO ANEXO

EFEITOS HISTOPATOLÓGICOS DA EXPOSIÇÃO CRÔNICA DE LARVAS DO PEIXE-REI MARINHO *ODONTESTHES ARGENTINENSIS* À FRAÇÃO SOLÚVEL DO PETRÓLEO EM ÁGUA (FSA)

Emeline P. Gusmão, Ricardo V. Rodrigues, Cauê B. Moreira, Luis André Sampaio, Kleber C. Miranda-Filho

Artigo a ser submetido à Revista AQUATIC TOXICOLOGY

Histopathological effects of chronic exposition of marine "pejerrey" *Odontesthes argentinensis* larvae to petroleum water-soluble fraction (WSF)

Emeline Pereira Gusmão[a], Ricardo Vieira Rodrigues[a], Cauê Bonucci Moreira[a], Luis André Sampaio[b], Kleber Campos Miranda-Filho[b]

a Programa de Pós-Graduação em Aqüicultura, Universidade Federal do Rio Grande, 96210-030, Querência, Rio Grande, RS, Brazil

b Instituto de Oceanografia, Universidade Federal do Rio Grande, 96210-030, Querência, Rio Grande, RS, Brazil

Corresponding author:

Kleber Campos Miranda Filho

Universidade Federal do Rio Grande

Instituto de Oceanografia, Laboratório de Piscicultura Marinha

Rua do Hotel, 02, Bairro Querência

96.210-030 – Rio Grande – RS – Brazil

Phone/FAX: + 55 53 3236-8042

E-mail: kleber08@gmail.com

ABSTRACT

The toxicological analysis of petroleum is usually performed on the basis of water-soluble fraction (WSF). The WSF contains a mixture of polycyclic aromatic hydrocarbons (PAHs), volatile hydrocarbons, phenols and heterocyclic compounds, considered deleterious to aquatic biota. Marine "pejerrey" *Odontesthes argentinensis* (Teleostei:Atherinopsidae) occurs from São Paulo, Brazil, to the South of Argentina, and it has a great commercial importance in local fisheries and a high potential for aquaculture. The aim of this work was to evaluate the histopathological effects in "pejerrey" larvae exposed to different concentrations of petroleum WSF. For the petroleum WSF, the total PAHs estimated by gas chromatography (GC) with a mass spectrometer (MS) was 197.83 μg L^{-1}, and 106.11 μg L^{-1} for total BTEX, estimated by GC with a flame ionization detector (FID) and a headspace Turbomatrix. For the acute toxicity test, 17 days old larvae were exposed to 5%, 10%, 20%, 40%, 60% and 100% of WSF, plus one control. After 96 hours of experiment, the LC_{50} (55% of WSF) and the safe level (5.5% of WSF) were estimated for the species. The chronic toxicity test was conducted with newly hatched larvae exposed to sublethal concentrations of WSF (2.5%, 5%, 10% and 20% of WSF), plus one control, in semi-static system for 21 days. Survival and growth were affected by petroleum WSF, and were significantly lower in the highest concentration. Several histopathological changes were found in the chronic toxicity test, which shows that histopathological biomarker was very sensitive in *O. argentinensis* larvae. The application factor used to estimate the safe level for toxic exposure to petroleum must be used with attention for "pejerrey" larvae.

KEYWORDS: petroleum WSF, "pejerrey", histopathology, lesion index.

1. 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). The population density in these regions is higher than in the continent and this overpopulation can cause an increase in dumping of xenobiotic compounds in coastal aquatic environments and consequent adverse effects to aquatic biota (Waldichuk, 1989; Tanabe and Tatsukawa, 1992; Kennish, 1997).

Chief among the contaminants affecting aquatic environments are organic carbon, heavy metals, synthetic organic compounds, petroleum hydrocarbons, industrial and urban sewage, radionuclides, litter and other sources (Kennish, 1997; Baird, 2002). Toxic effects of petroleum are well recognized, due to its growing demand by mankind (Clark, 2001).

Petroleum water-soluble fraction (WSF) is the aqueous phase enriched with complex components like aromatic and aliphatic hydrocarbons, and heterocyclic compounds containing nitrogen or sulfur (Ziolli and Jardim, 2002; Akaishi et al., 2004). The WSF is the portion that enters in the aquatic environment and special attention is required to monocyclic aromatic hydrocarbons, such as BTEX compounds (benzene, toluene, ethylbenzene and xylene) because they are highly hydrosoluble, they can be absorbed by organisms, and therefore toxic to biota (Stephens et al., 1997; Dórea et al., 2007). The polycyclic aromatic hydrocarbons (PAHs) are not particularly water soluble and their concentrations in aquatic environments remain low, despite the increasing levels in the aquatic environment due to anthropogenic inputs (Hylland, 2006).

However, the PAH fraction is lipid soluble and tend to bioconcentrate in aquatic animals (Kennish, 1997).

According to Monserrat et al. (2006), biomarkers are measures of body fluids, cells, tissues or even behavioural parameters that may detect sublethal biological effects of pollutants. Previous reports have been conducted using histopathology as biomarker, including toxicological investigations with petroleum and refined products (Myers et al., 1998; Grinwis et al., 2000; Simpson et al., 2000; Nero et al., 2006a,b).

The use of organisms as bioindicators and biomonitors of pollution is well-consolidated in environmental contamination monitoring activities (Claisse, 1989; Wester et al., 2002). Some fish species already used in aquatic toxicology can be easily obtained from aquaculture sources, such as rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Therefore, in addition to the food production with high protein content (FAO, 2006), aquaculture may provide aquatic organisms to ecotoxicological assays in environmental monitoring activities.

Different studies have been developed to assess the petroleum WSF toxicity in fish (Lockhart et al., 1996; Omoregie and Ufodike, 2000; Dede and Kaglo, 2001; Akaishi et al., 2004) and they have shown that fish are reliable bioindicators of PAHs contamination in marine samples (Gagnon and Holdway, 1999; Martínez-Gómez et al., 2006; Morales-Caseles et al., 2006; Ramachandran et al., 2006). Negative effects of petroleum WSF were reported upon fish growth and metabolism, due to pathologies in organs such as liver, gills and kidney (Khan, 2003; Davoodi and Claireaux, 2007). In embryos, eggs and larvae of marine fish, it was found that the WSF has mutagenic and teratogenic effects, and other deleterious responses during the development, such as gill and liver alterations (Brand et al., 2001; Middaugh et al., 2002; Pollino and Holdway,

2002). Furthermore, fish early life stages are in general more sensitive to pollutantsand generally are unable to avoid oil slicks because of their low mobility. Sublethal effects include abnormal development, reduced growth and cellular abnormalities (Kennish, 1997).

For several years, some species of silversides have been used in ecotoxicological studies, like the inland silverside *Menidia beryllina* (Teleostei: Atherinopsidae), approved by the USEPA (United States Environmental Protection Agency) as a marine and estuarine species for acute and chronic toxicity tests (Anderson et al., 1974; Hemmer et al., 1992; Al-Yakoob et al., 1996). The "pejerrey" *Odontesthes argentinensis* (Valenciennes, 1835) (Teleostei: Atherinopsidae) is distributed from São Paulo, Brazil, to the south of Argentina (Brian and Dyer, 2006), and it has great importance in local fisheries. Several studies have been conducted in southern Brazil with "pejerrey", aiming its introduction in aquaculture activities (Tesser and Sampaio, 2001; Sampaio, 2006). Then, there is a great potential for the use of this species to aquaculture and ecotoxicological essays, and even for biomonitoring studies.

Although there are no records of significant oil spills in the southern coast of Brazil, there are risks due to navigation activities and the presence of companies of the oil industry, working with refining, storage and transportation around this area. The "pejerrey" uses the southern coast for reproduction and growth, and it is an important species for fisheries and potential candidate for aquaculture. Thus, the present article has the objective to evaluate the histopathological sublethal effects on early life stages of marine "pejerrey" *O. argentinensis* after chronic exposition to petroleum WSF. In order to assist the interpretation of the gill histopathological effects, an index of injury was adapted to the species.

2. MATERIAL AND METHODS

2.1. SAMPLING AND MAINTENANCE OF ORGANISMS

Natural fertilized eggs of *O. argentinensis* were collected at the Cassino beach (32°30'S, 52°30'W), Rio Grande, RS, Brazil, and immediately transported to the Laboratory of Marine Fish Culture belonged to Federal University of Rio Grande (FURG). The eggs were incubated in a tank of 50 L with filtered (5 µm) seawater at salinity 30, temperature of 23°C and dissolved oxygen $\geq$ 6.2 mg L^{-1}. Soon after hatching, larvae were transferred to round tanks with capacity for 50 L, with constant aeration, 50% daily renewal of the water and removal of food rests and feces. The larvae were fed with newly hatched *Artemia* sp nauplii, according to the protocol of larviculture for this species (Sampaio, 2006), until the development of the organs, 17 days after hatch (9 mg) for the acute toxicity test. Newly hatched larvae (1.9 mg) were used to chronic toxicity test.

2.2. PETROLEUM WSF PREPARATION

In order to obtain the petroleum WSF, one part of a Brazilian heavy crude oil (source: Petrobras, 19° API) was mixed with nine parts of filtered seawater in a 5 L sealed "Mariotti" flask, during approximately 22 hours with a magnetic stirrer (Quimis, Q241, Brazil) at room temperature using a fume hood, without light exposure, according to the method proposed by Anderson et al. (1974) with some modification. After about 2 hours of resting, the aqueous phase was separated from the oily phase.

The WSF obtained was then diluted in different concentrations, using filtered seawater, to achieve specified concentrations for acute and chronic toxicity tests.

2.3. QUALI-QUANTITATIVE ANALYSES OF WSF

A 100% petroleum WSF sample was collected (1 L aliquot) and analyzed qualitative and quantitatively for total petroleum hydrocarbons (TPH) determination (ISATEC Laboratories). The 16 PAHs (∑16 PAHs) recommended by US EPA were determined by gas chromatography according to US EPA Method 8270D, 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, using a Perkin-Elmer (Clarus 500) GC with a flame ionization detector (FID) and a headspace Turbomatrix HS 40 sampler.

2.4. TOXICITY TESTS

2.4.1. ACUTE TOXICITY TEST

The acute toxicity test was conducted as a completely randomized design in which larvae of 17 days after hatching (1.9 mg) were exposed to petroleum WSF in 1 L beakers, with no aeration, for 96 hours. The exposition was semi-static (daily renewal of 50%), due to the small size of the larvae and to diminish the quantity of WSF needed to the experiment. Larvae with 17 days after hatching were chosen because major organs such as gills, kidney and liver in the “pejerrey” larvae are functional around 15 days

after hatching. The concentrations tested were produced from petroleum WSF dilutions in filtered saltwater: 5%, 10%, 20%, 40%, 60% and 100%, plus a control (with no WSF). All treatments were done in triplicate, totaling 30 individuals per treatment. Before renewing the experimental *millieu*, physical-chemical parameters (temperature, dissolved oxygen, pH and salinity) were measured, and dead fish (with no observable movement) were counted daily. Salinity was measured with a hand refratometer (Atago, S/Mill, Japan), pH with an electronic benchtop 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 (LC_{50}) was estimated using the software Trimmed Spearman Karber method (Hamilton et al., 1977). The safe level (used to predict concentrations, below this safe level, in which the individuals can be exposed with no lethal or sublethal effects) was estimated using the LC_{50} 96h value and an application factor (10% of the LC_{50} value) proposed by Sprague (1971).

2.4.2. CHRONIC TOXICITY TEST

The chronic toxicity test was also completely randomized in which newly hatched larvae were exposed semi-statically to sublethal concentrations of petroleum WSF, during 21 days, when significative differences in the growth could be noted. The concentrations tested were 2.5%, 5%, 10% and 20% of WSF, plus a control group (no WSF added). All treatments were conducted with four replicates, totaling 60 individuals per treatment. Physical-chemical parameters were measured (see item 2.4.1) and dead fish were counted daily. A biometry was carried out at the beginning of the test with

newly hatched larvae, and in order to verify larval growth, new biometries for all treatments were performed weekly, using larvae with 7, 14 and 21 days after hatching (*n* per treatment = 8). The larvae were anesthetized with benzocaine (50 ppm) in order to measure weight and length, and after this they were collected for histopathological analysis (see item 2.5). Results of growth and survival, as well as physical-chemical parameters were analyzed using one-way ANOVA for the significant differences ($p < 0.05$) between treatments, and when they were found, the Tukey test was performed using the software Statistica 6.0 (StatSoft). Data are presented as mean ± standard error.

2.5. HISTOLOGICAL PROCEDURE

All larvae proceeding from the chronic test biometries were collected (7, 14 and 21 days after hatching, *n* per treatment = 8). They were anesthetized with benzocaine (30 ppm) and fixed in Bouin's liquid (12-24 hours) for routine histological processing with paraffin embedding (entire larvae), microtomy with 7 µm and staining with hematoxylin-eosin. The slides were mounted with synthetic Canada balsam, observed with optical microscope (Olympus BH-2) and photographed with digital camera (Olympus Camedia C-5060). All the organs of the larvae were analyzed, with special attention to the gills, liver and kidney.

2.6. HISTOPATHOLOGICAL EVALUATION

Histopathological changes were evaluated according to the protocol proposed by Bernet et al. (1999) and adapted to the species of this study. Different branchial alterations are listed in Table 1, and are classified into three importance factors: 1 – minimal pathological importance, the lesion is reversible as the exposure to toxicant ends; 2 – moderate pathological importance, the lesion is reversible in some cases if the exposure ends; and 3 – marked pathological importance, the lesion generally is irreversible, leading to partial or total loss of the organ function. Every alteration was assessed using a score ranging from 0 to 6, depending on the extent and degree of the alteration: 0 – unchanged, 2 – mild occurrence, 4 – moderate occurrence, and 6 – severe occurrence. Intermediate values were also considered.

Table 1.

Using importance factors and score values, a gill lesion index (I_G) was calculated as: $I_G = \Sigma_{rp}\Sigma_{alt}$ (aw), where rp = reaction pattern, alt = alteration, a = score and w = importance factor. This index represents the degree of damage to the gills. It is the sum of the multiplied importance factors and score values of all changes found in the gills, which allows the comparison between the degree of damage of this organ in different individuals. A high index indicates a high degree of damage. This index was also analyzed following one-way ANOVA and Tukey test ($p < 0,05$).

3. RESULTS

3.1. WSF QUALI-QUANTITATIVE ANALYSIS

The total concentrations of PAHs and BTEX were estimated at 13.55 µg L^{-1} and 4,843.02 µg L^{-1}, respectively, and the total petroleum hydrocarbons (TPH) was calculated at 4,856.57 µg L^{-1}.

Table 2

3.2. ACUTE TOXICITY TEST

Dissolved oxygen concentration and pH were significantly lower for 100% of WSF than other dilutions (Table 2). In this concentration, all fish died before 24 h of exposure.

Table 3

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

3.3. CHRONIC TOXICITY TEST

3.3.1. PHYSICAL-CHEMICAL PARAMETERS

Mean values of temperature, dissolved oxygen and pH for each concentration of petroleum WSF are presented in Table 3. These parameters were not affected by different dillutions of WSF.

Table 4

3.3.2. SURVIVAL AND GROWTH

Table 3 also shows that the survival of larvae was affected by increasing concentrations of petroleum WSF. Survival in the highest concentration was significantly lower ($P < 0.05$) than the control and 5% of WSF, and was not different from 2.5% and 10% of WSF.

Figure 1

After 21 days of exposure, the animals exposed to 20% of petroleum WSF were significantly lower in weight than in other treatments (Figure 1).

3.3.3. HISTOPATHOLOGICAL EVALUATION

3.3.3.1. GILLS

The gills were affected with the exposure to petroleum WSF and the damage was increasing over the period in which the larvae were exposed, especially in higher concentrations.

The normal structure of the gills can be seen in Figure 2A, in which the branchial arch supports the primary lamellae from where the secondary lamellae part. Secondary lamellae are constituted by capillars, pillar cells and respiratory epithelium. In the control treatment, in all periods analyzed, some abnormalities were observed, such as, epithelial lifting and secondary lamellae fusion. The lesion index (I_G) was 0.4, 2.6 and 4.9 to 7, 14 and 21 days, respectively.

In the 2.5% of petroleum WSF concentration, aneurisms with pillar cell rupture (Fig. 2B), lifting of respiratory epithelium (Fig. 2C), fusion of secondary lamellae and edema were observed in "pejerrey" larvae at 14 days. One week later a few cases of hyperplasia of interlamellar epithelium were observed also. The I_G determined were 2.8, 16.6 and 15.7 for 7, 14 and 21 days, respectively.

Figure 2

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

In the concentration of 10% of petroleum WSF, aneurisms, 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.

Figure 3

The lesion index (I_G) showed significant differences among treatments, as shown in the graph of Figure 3. For the control, the 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.

3.3.3.2. LIVER

The liver showed changes in accordance with the WSF concentration and the time of exposure, but not as marked as the branchial changes.

The structure of normal liver is shown in Figure 4A, in which is perceived hepatocytes arranged in cords with a single layer of cells lining each sinusoid (Akiyoshi and Inoue, 2004), with energy stocks and uniform nuclei with a single nucleolus.

Figure 4

Among the most frequent alterations, there was the intense cytoplasm basophilia of the hepatocytes and nuclear changes such as hypertrophy, karyorrhexis and karyopyknosis. 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 WSF. The other abnormalities described were found only in concentrations of 10% and 20% of petroleum WSF (Fig. 4B and 4C).

The reserve of lipids and carbohydrates does not appear to have been affected by WSF exposure, because in the control group, as well as in other concentrations, liver with little or no reserves were observed, and also highly vacuolated. There were no changes in biliary ducts of livers analyzed.

3.3.3.3. KIDNEY

In the Figure 5A, it is possible to observe the normal structure of the anterior kidney at 21 days after hatching in the control group. The kidney is formed by renal tubules, glomeruli and hematopoietic tissue.

Figure 5

Nuclear alterations were observed in renal tubules, such as hypertrophy, karyorrhexis and karyopycnosis, in the concentrations of 10% and 20% (Fig. 6B). Clear spaces between tubular epithelial cells were also observed in the posterior kidney (Fig. 6C), meaning a disequilibrium in the structure of the tubules. In these concentrations, a lower amount of hematopoiethic tissue was also observed, possibly due to several hemorrhage in the gills and constant need of blood cells reposition.

4. DISCUSSION

Toxicological results are difficult to compare because different types of petroleum and different ways to prepare the WSFs are used in toxicity studies, and this can cause significant differences in the results (Singer et al., 2000; Ziolli and Jardim, 2002). In the literature, there is no uniformity in the experimental steps utilized by different authors in the WSF preparation and this results in considerable difficulties to compare experimental data.

Brand et al (2001) found the same proportion of hydrocarbons in the WSF of North Slope crude oil. BTEX correspond to the major compounds in the WSF (94% of TPHs) and naphthalene corresponds to 1% of TPHs. Barron et al. (1999) and Neff et al.

(2000) corroborate the major BTEX composition in the WSF of this study, being the main responsible for the toxicity of marine fish, although its high volatibility.

Lockhart et al. (1996) studied the chronic toxicity effects in juveniles of rainbow trout exposed to Norman Wells crude oil WSF for 55 days. Tested concentrations (0.15, 0.39 and 1.51 mg L^{-1} TPH) caused mortality and some harmful effects such as fin erosion and increase of water content in the body, with higher mortality in the highest concentration. TPH values in the present study were higher than those, corraborating the toxic effects related to hydrocarbons observed in both studies.

Brand et al. (2001) exposed pink salmon fry to TPH concentrations varying from 25 to 348 µg L^{-1} for over 10 days and found some deleterious effects in the gills. Neff et al. (2000) found TPH concentrations varying from 0.008 to 38.31 mg L^{-1} for different Australian crude oils. Woodward et al. (1981), working with Wyoming crude oil, suggests that a safe concentration to avoid biological effects on fish must be less than 24 µg L^{-1}, which is much less than the measured concentrations of TPH in the Brazilian crude oil WSF.

Different species have distinct tolerances to crude oils, and marine fish seems to be more tolerant than freshwater fish (Ramachandran et al., 2006/; Schukla et al., 2007). The LC_{50}-96h value for *Menidia beryllina* silversides were calculated between 32 and 88% of the WSF of three different crude oils (Neff et al., 2000), not different from the present investigation (55% of WSF).

In addition to toxicity tests, we chose the histopathological study in order to observe chief effects generated to gill, liver, kidney and other tissues by petroleum exposure. Furthermore, a gill lesion index was adapted in order to assist the interpretation of the gill damage during 21 days of chronic test.

Previous reports described injury in gill exposed to petroleum components (Brand et al., 2001; Akaishi et al., 2004; Morales-Caselles et al., 2006; Nero et al., 2006a,b; Simonato et al., 2007). The most commonly described are: lifting of respiratory epithelium, tissue necrosis, hyperplasia, hypertrophy, hemorrhage and aneurism. Some of these lesions are irreversible, and after a chronic exposure to toxicant, can cause death of the organism by imbalances in gill activities such as gas exchanges, ionorregulation, maintenance of acid-base balance and excretion of nitrogen compounds (Di Giulio and Hinton, 2008).

Morales-Caselles et al. (2006) reported some histopathological effects in the gills of juveniles of *Sparus aurata* exposed to sediment contaminated by *Prestige* oil spill, such as shortaning of secondary lamellae, hypertrophy, hyperplasia, necrosis, loss of epithelial cells, fusion of secondary lamellae and presence of edematous areas in the distal portion of lamellae. As in the present study, gills were shown to be the most damaged tissue, showing different lesions.

Akaishi et al. (2004) also reported histopathological effects on the gills of a freshwater fish (*Astyanax* sp), like disorganization of secondary lamellae and telangiectasia, after acute exposure to Campos Bay (Brazil) crude oil WSF. Gills of *Oncorhynchus mykiss* exposed to a petroleum WSF with 0.043 and 0.350 mg L^{-1} of TPHs for 30 days showed epithelial lifting and damage of secondary lamellae, associated with cellular hypertrophy (Rudolph et al, 2001). Khan (2003) also observed hyperplasia and hypertrophy of the lamellar epithelium in three species of marine flatfishes from an area near to an oil refinery. These alterations can be related to petroleum hydrocarbons.

In this investigation, the gill lesion index was significantly higher for fish exposed to higher concentrations of petroleumWSF, suggesting that higher number of injures and more severe lesions were found in the gills of these fish, considerably increasing the index. The higher the index, the greater the degree of damage in the gills of the larvae, which can be correlated to the mortality and lower growth of most affected larvae, which probably needed to conduct its available energy require for osmorregulation and maintenance of homeostasis balance, instead of using this energy for growth and energy reserves.

In the gills, the lesions begin with the respirathory epithelium lifting due to edema, causing the loss of cellular junctions of the epithelium. Functionally, the edema considerably increases the barrier between water and blood, with serious respirathory alterations. The pillar cell and endothelial cell rupture follows these alterations causing loss of erythrocytes and problems in the branchial circulation, such as telangiectasia. This alteration is incompatible with life, and it is not reversible, due to its gravity (Romano and Cueva, 1988).

Romano and Cueva (1988) also report that in some cases the hyperplasia can be so diffuse that there is no way for the water to pass, and this is a reactive and adaptative phenomenon of the epitelium to the exposure to toxicants. Although, if it reaches a high extension, inhibits the gas diffusion. All these gill problems lead to the lack of oxygen necessary to the metabolism of the animal.

Lamellar fusion of gills could be a protective effect, in order to diminish the gill surface area exposed to the toxicant (Mallat, 1985). More diffuse hyperplastic primary and secondary lamellae (not focal hyperplasia) suggests that the etiologic agent in the present study was chemical (Brand et al, 2001).

The alterations found in the liver have also been reported in the literature (Brand et al., 2001; Stehr et al., 2003; Akaishi et al., 2004; Morales-Caselles et al., 2006; Simonato et al., 2007) to several petroleum compounds. One of the most common change is the hypertrophy of hepatocytes or nucleus, although this can 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 (Hibiya, 1982), these cells can be hypertrophied. 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, and few energy reserves to be found in these cells. 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 number of cases found in larvae exposed to higher concentrations of WSF in the chronic test.

Morales-Caselles et al (2006) also reported for *S. aurata* juveniles some histopathologies in the liver, such as vacuolization of hepatocytes, necrosis and an increased cytoplasmic basophilia related to increase of PAHs. An increased basophilia in hepatocyte cytoplasms 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, were also found several cases of intense basophilia in hepatocytes, which may be related to intense activity of these cells, including detoxification.

Akaishi et al (2004) reported some 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, and decreases the functional number of cells in the tissue with deleterious consequences to the organ function. Hepatocytes showing dense and homogenous cytoplasm with disappearance of cellular limits suggest alterations in the distribution of organelles, and the cellular hypertrophy can affect the metabolism of hepatocytes involved in important functions like storage of nutrients and xenobiotic detoxification.

Few studies have been performed with renal changes as biomarkers of environmental pollution. Silva and Martinez (2007) reported many pathological changes in the kidney of a freshwater fish known as "lambari" (*Astyanax altiparanae*) exposed to urban wastewater. Similar alterations were found in this study. As the kidney receives a great part of post-branchial blood, and abnormalities were found in the gills, kidney alterations can be also due to the exposure to toxic compounds.

The histopathological effects observed in the present investigation are nonspecific and can be due to a variety of pollutants, and therefore only indicating the general quality of the environment rather than specific types of pollutants (Au, 2004). The cause-effect relationships and detailed mechanisms leading to the development of most pathological symptoms are not generally clear. In contrast, certain hepatic lesions in fish have been well correlated with contaminant exposure (Au, 2004).

The importance of using chronic bioassays has been proved, 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).

5. CONCLUSIONS

The present study demonstrated that sublethal exposure to the WSF of crude oil results in a significant stress response as indicated by altered structure of liver, kidney and gill tissues. The "pejerrey" larvae can suffer irreversible damage when exposed to toxic concentrations of petroleum WSF. The histopathological biomarker proved to be very sensitive in relation to acute and chronic petroleum exposure. The application factor used to estimate the safe level for toxic exposure to petroleum mus be used with attention for "pejerrey" larvae. 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. There is, however, a need to increase our understanding about physiological and genetic effects of petroleum exposure. Future studies are required to investigate the effects of sublethal concentrations of petroleum on different life stages of the "pejerrey" *O. argentinensis*.

REFERENCES

Akaishi, F.M., Assis, H.C.S., Jakobi, S.C.G., Eiras-Stofella, D.R., St-Jean, S.D., Courtenay, S.C., Lima, E.F., Wagener, A.L.R., Scofield, A.L., Ribeiro, C.A.O., 2004. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax* sp.) after Waterborne and acute exposure to water soluble fraction (WSF) of crude oil. Arch. Environ. Contam. Toxicol. 46, 244-253.

Akiyoshi, H., Inoue, A., 2004. Comparative histological study of teleost livers in relation to phylogeny. Zool. Sci. 21, 841-850.

Al-Yakoob, S.N., Gundersen, D., Curtis, L., 1996. Effects of the water-soluble fraction of partially combusted crude oil from Kuwait's oil fires (from desert storm) on survival and growth of the marine fish *Menidia beryllina*. Ecotoxicol. Environ. Saf. 35, 142-149.

Anderson, J.W., Neff, J.M., Cox, B.A., Tatem, H.E., Hightower, G.M., 1974. Characteristics of dispersions and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. Mar. Biol. 27, 75-88.

Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar. Pol. Bull. 48, 817-834.

Baird, C., 2002. Química ambiental. Bookman, Porto Alegre.

Barron, M.G., Pochatsky, T., Ogle, S., Ricker, R.W., 1999. Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? Aq. Toxicol. 46, 253-268.

Bernet, D., Schmidt, H., Meier, W., Burckhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis. 22, 25-34.

Brand, D.G., Fink, R., Bengeyfield, W., Birtwell, I.K., Mcallister, C.D., 2001. Salt-water acclimated pink salmon fry (*Oncorhynchus gorbusha*) develop stress-related visceral lesions after 10-day exposure to sublethal concentrations of the water-soluble fraction of North Slope crude oil. Toxicol. Pathol. 29 (5), 574-584.

Brian, S., Dyer, H., 2006. Systematic revision of the South American silversides (Teleostei, Atheriniformes). Biocell 30 (1), 69-88.

Claisse, D., 1989. Chemical contamination of French coasts: The results of a ten years Mussel Watch. Mar. Pollut. Bull. 20, 523-528.

Clark, R., 2001. Marine pollution. Oxford University Press, Oxford.

Davoodi, F., Claireaux, G., 2007. Effects of exposure to petroleum hydrocarbons upon the metabolism of the common sole *Solea solea*. Mar. Pol. Bul. 54, 928-934.

Dede, E.B., Kaglo, H.D., 2001. Aqua-toxicological effects of water soluble fractions (WSF) of diesel fuel on *O. niloticus* fingerlings. J. Appl. Sci. Environ. Manag. 5 (1), 93-96.

Di Giulio, R.T., Hinton, D.E., 2008. Toxicology of fishes. CRC Press, USA, 1099p.

Dórea, H.S., Bispo, J.R.L., Aragão, K.A.S., Cunha, B.B., Navickiene, S., Alves, J.P.H., Romão, L.P.C., Garcia, C.A.B., 2007. Analysis of BTEX, PAHs and metals in the oilfield produced water in the State of Sergipe, Brazil. Microchem. J. 85, 234-238.

FAO – Food and Agriculture Organization of the United Nations, 2006. The state of world fisheries and aquaculture - 2006. Rome, Italy.

Gagnon, M.M., Holdway, D.A., 1999. Metabolic enzyme activities in fish gills as biomarkers of exposure to petroleum hydrocarbons. Ecotoxicol. Environ. Saf. 44, 92-99.

Grinwis, G., Besselink, H., Van Den Brandhof, E., Bulder, A., Engelsma, M., Kuiper, R., Wester, P., Vaal, M., Vethaak, A., Vos, J., 2000. Toxicity of TCDD in European flounder (*Platichthys flesus*) with emphasis on histopathology and cytochrome P450 1A induction on several organ systems. Aquat. Toxicol. 50, 387-401.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Karber Method for estimating median Lethal Concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714-719.

Hemmer, M., Middaugh, D., Comparetta, V., 1992. Comparative acute sensitivity of larval topsmelt, Atherinops affinis, and inland silverside, *Menidia beryllina*, to 11 chemicals. Environ. Toxicol. Chem. 11, 401-408.

Hibiya, T., 1982. An atlas of fish histology – Normal and pathological features. Kodansha, Japan.

Hinton, D., Laurén, D., 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. Am. Fish. Soc. Symp. 8, 51-66.

Hylland, K., 2006. Polyciclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems. J. Toxicol. Environ. Health, Part A 69, 109-123.

Khan, R.A., 2003. Health of flatfish from localities in Placentia Bay, Newfoundland, contaminated with petroleum and PCBs. Arch. Environ. Contam. Toxicol. 44, 485-492.

Kennish, M., 1997. Practical handbook of estuarine and marine pollution. CRC Press, USA.

Lockhart, W.L., Duncan, D.A., Billeck, B.N., Dannell, R.A., Ryan, M.J., 1996. Chronic toxicity of the "water soluble fraction" of Norman Wells crude oil to juvenile fish. Spill Sci. Technol. Bull. 3 (4), 259-262.

Mallat, J., 1985. Fish gill structural changes induced by toxicants and other irritants. A statistical review. Can. J. Fish Aquat. Sci. 42, 630-648.

Martínez-Gómez, C., Campillo, J.A., Benedicto, J., Fernández, B., Valdés, J., García, I., Sanchez, F., 2006. Monitoring biomarkers in fish (*Lepidorhombus boscii* and

Callionymus lyra) from the northern Iberian shelf after the Prestige oil spill. Mar. Pollut. Bull. 53, 305-314.

Middaugh, D.P., Chapman, P.J., Shelton, M.E., Mckenney Jr, C.L., Courtney, L.A., 2002. Effects of fractions from biodegraded Alaska North Slope Crude Oil on embryonic inland silversides, *Menidia beryllina*. Arch. Environ. Contam. Toxicol. 42, 236-243.

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Amado, L.L., Martins, C.M.G., Pinho, G.L.L., Chaves, I.S., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2006. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comp. Biochem. Physiol., Part C: In press.

Morales-Caselles, C., Jiménez-Tenorio, N., Canales, M.L.G., Sarasquete, C., Delvales, T.A., 2006. Ecotoxicity of sediments contaminated by the oil spill associated with the tanker "Prestige" using juveniles of the fish *Sparus aurata*. Arch. Environ. Contam. Toxicol. 51, 652-660.

Myers, M.S., Johnson, L.L., Hom, T., Collier, T.K., Stein, J.E., Varanasi, U., 1998. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: relationships with other biomarkers of contaminant exposure. Mar. Environ. Res. 45(1), 47-67.

Neff, J.M., Ostazeski, S., Gardiner, W., Stejskal, I.. 2000. Effects of weathering on the toxicity of three offshore Australian crude oils and a diesel fuel to marine animals. Environ. Toxicol. Chem. 19(7), 1809-1821.

Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., Dixon, D.G., 2006a. The effects of salinity on naphthenic acid toxicity to yellow perch: gill and liver histopathology. Ecotox. Environ. Saf. 65, 252-264.

Nero, V., Farwell, A., Lister, A., Van Der Kraak, G., Lee, L.E.J., Van Meer, T., Mackinnon, M.D., Dixon, D.G., 2006b. Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Cassius auratus*) exposed to oil sands process-affected water. Ecotoxicol. Environ. Saf. 63, 365-377.

Omoregie, E., Ufodike, B.C., 2000. Effects of water soluble fractions of crude oil on growth of the Nile tilapia, *Oreochromis niloticus* (L.). Bull. Environ. Contam. Toxicol. 64, 601-605.

Pollino, C.A., Holdway, D.A., 2002. Toxicity testing of crude oil and related compounds using early life stages of the crimson-spotted rainbowfish (*Melanotaenia fluviatilis*). Ecotox. Environ. Saf. 52, 180-189.

Ramachandran, S.D., Sweezey, M.J., Hodson, P.V., Boudreau, M., Courtenay, S.C., Lee, K., King, T., Dixon, J.A., 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Mar. Pollut. Bull. 52, 1182-1189.

Romano, L.A., Cueva, F., 1988. Lesiones histologicas branquiales atribuibles a toxicos en *Odontesthes bonariensis* (Cuv. y Val., 1835) (Pisces, Atherinidae). Rev. Asoc. Cienc. Nat. Litoral 19 (2).

Rudolph, A., Yanez, R., Troncoso, L., 2001. Effects of exposure of *Oncorhynchus mykiss* to the water accomodated fraction of petroleum hydrocarbon. Bull. Environ. Contam. Toxicol. 66, 400-406.

Sampaio, L.A., 2006. Production of "pejerrey" *Odontesthes argentinensis* fingerlings: A review of current techniques. Biocell 30 (1), 121-123.

Sarasquete, C., Gutiérrez, M., 2005. New tetrachronic VOF stain (type iii-gs) for normal and pathological fish tissues. Eur. J. Histochem. 49, 105-114.

Schukla, P., Gopalani, M., Ramteke, D.S., Wate, S.R., 2007. Influence of salinity on PAH uptake from water-soluble fraction of crude oil in *Tilapia mossambica*. Bull. Environ. Contam. Toxicol. 79, 601-605.

Silva, A.G., Martinez, C.B.R., 2007. Morphological changes in the kidney of a fish living in an urban stream. Environ. Toxicol. Pharmacol. 23, 185-192.

Simonato, J.D., Guedes, C.L.B., Martinez, C.B.R., 2007. Biochemical, physiological, and histological changes in the neotropical fish *Prochilodus lineatus* exposed to diesel oil. Ecotoxicol. Environ. Saf. 69 (1), 112-120.

Simpson, M.G., Parry, M., Kleinkauf, A., Swarbreck, D., Walker, P., Leah, R.T., 2000. Pathology of the liver, kidney and gonad of flounder (*Platichthys flesus*) from a UK estuary impacted by endochrine disrupting chemicals. Mar. Environ. Res. 50, 283-287.

Singer, M.M., Aurand, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L., Tjeerdema, R.S., 2000. Standardization of the preparation and quantification of water-accomodated fractions of petroleum for toxicity testing. Mar. Pol. Bull. 40, 1007-1016.

Sprague, J.B., 1971. Measurement of pollutant toxicity to fish – III Sublethal effects and safe concentrations. Water Res. 5, 245-266.

Stehr, C.M., Myers, M.S., Johnson, L.L., Spencer, S., Stein, J.E., 2003. Toxicopathic liver lesions in English sole and chemical contaminant exposure in Vancouver Harbor. Can. Mar. Environ. Res. 57, 55-74.

Stephens, S.M., Alkindi, A.Y.A., Waring, C.P., Brown, J.A., 1997. Corticosteroid and thyroid responses of larval and juvenile turbot exposed to the water-soluble fraction of crude oil. J. Fish Biol. 50, 953-964.

Tanabe, S., Tatsukawa, R., 1992. Chemical modernization and vulnerability of cetaceans: increasing toxic threat of organochlorine contaminants. In: Persistent pollutants in marine ecosystems. Oxford: Pergamon Press, p. 161-177.

Tesser, M., Sampaio, L.A., 2001. Growth of pejerrey larvae (*Odontesthes argentinensis*) on different prey densities. Atlântica 23, 153-159.

US EPA – United States Environmental Protection Agency. Method 8015B. Washington, DC.

US EPA – United States Environmental Protection Agency. Method 8270D. Washington, DC.

Vethaak, A.D., Wester, P.W., 1996. Diseases of flounder *Platichthys flesus* in Dutch Coastal and estuarine waters with particular reference to environmental stress factors. Dis. Aquat. Org. 26, 99-116.

Waldichuk, M., 1989. The state of pollution in the marine environment. Mar. Pollut. Bull. 20, 598-602.

Wester, P.W., Van Der Ven, L.T.M., Vethaak, A.D., Grinwis, G.C.M., Vos, J.G., 2002. Aquatic toxicology: opportunities for enhancement through histopathology. Environ. Toxicol. Pharmacol. 11, 289-295.

Woodward, D.F., Mehrle Jr., P.M., Mauck, W.L., 1981. Accumulation and sublethal effects of a Wyoming crude oil in cutthroat trout. Trans. Am. Fish. Soc. 110, 437-445.

Ziolli, R.L., Jardim, W.F., 2002. Operational problems related to the preparation of the seawater soluble fraction of crude oil. J. Environ. Monit. 4, 138-141.

Table 1. Gill histopathological alterations in "pejerrey" *Odontesthes argentinensis* larvae and their respective importance factors (adapted from Bernet et al., 1999).

Reaction pattern	**Alteration**	**Importance factor**
Circulatory disturbances	Hemorrhage/hyperemia/aneurysm	1
	Intercellular oedema	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
	Necrosis	3
Progressive changes	Respiratory epithelium hypertrophy	1
	Interlamellar epithelium hyperplasia	2
Inflammation	Infiltration	2

Table 2. Concentration of total monocyclic aromatic hydrocarbons (BTEX) and polycyclic aromatic hydrocarbons (PAHs) in the water soluble fraction (WSF) of Brazilian petroleum.

Hydrocarbon	**Concentration (µg L^{-1})**
Benzene	1,957.78
Toluene	1,845.30
Ethylbenzene	63.21
Xylene	976.73
Total BTEX	4,843.02
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
Total petroleum hydrocarbons (TPHs)	4,856.57

* nd = not detected

Table 3. Physical-chemical parameters of different concentrations of petroleum WSF used in acute toxicity test with *Odontesthes argentinensis* larvae. Significant differences are shown by different letters (P<0.05).

Concentration	**Temperature (°C)**	**O_2 (mg L^{-1})**	**pH**
Control	22.7 ± 0.4^{ab}	5.54 ± 0.35^{a}	7.93 ± 0.11^{a}
5%	22.8 ± 0.2^{a}	5.33 ± 0.19^{a}	7.90 ± 0.05^{a}
10%	22.5 ± 0.2^{ab}	5.43 ± 0.33^{a}	7.92 ± 0.08^{a}
20%	22.6 ± 0.3^{ab}	5.51 ± 0.34^{a}	7.92 ± 0.06^{a}
40%	22.6 ± 0.1^{ab}	5.31 ± 0.36^{a}	7.88 ± 0.06^{a}
60%	22.6 ± 0.28^{ab}	5.5 ± 0.31^{a}	7.87 ± 0.09^{a}
100%*	22.3^{b}	3.72^{b}	7.61^{b}

* There is only the initial value because all the fish died before 24 h of experiment.

Table 4. Survival of "pejerrey" *Odontesthes argentinensis* larvae and physical-chemical parameters of petroleum WSF concentrations used in the chronic toxicity test, after 21 days of exposure to WSF. Different letters indicate statistical significant differences ($P<0.05$).

Concentration	**Survival (%)**	**Temp.(ºC)**	**O_2 (mg L^{-1})**	**pH**
Control	88.6 ± 6.81 [a]	22.7 ± 0.5	6.32 ± 0.83	7.85 ± 0.52
2.5%	70.4 ± 6.82 [ab]	22.6 ± 0.4	6.26 ± 1.06	7.85 ± 0.50
5%	84.1 ± 10.08 [a]	22.7 ± 0.3	6.33 ± 1.01	7.86 ± 0.47
10%	65.9 ± 2.27 [ab]	22.7 ± 0.3	6.42 ± 1.10	7.91 ± 0.46
20%	36.4 ± 11.13 [b]	22.6 ± 0.3	6.52 ± 1.07	7.94 ± 0.48

FIGURE CAPTIONS

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

Figure 2. Gill sections from "pejerrey" *Odontesthes argentinensis* larvae. A. Branchial arches (ga) of a larva of 21 days in the control group, with primary (pl) and secondary (sl) lamellae with normal structure. 400x. B. Secondary lamellae with aneurism (arrows) in a larva of 14 days exposed to 2.5% of petroleum WSF. 400x. C. Secondary lamellae showing lifting of the respiratory epithelium (arrow) in 21 days old larvae exposed to 2.5% of petroleum WSF. 1000x. D. Hyperplasia of interlamellar epithelium (arrows) in 21 days old larvae in the concentration of 5% of petroleum WSF. 400x. E. Branchial cavity of a larva of 14 days exposed to 10% of petroleum WSF, showing hyperplasia of the cavity epithelium (arrow); pg – pseudobranchia. 200x. F. Gill with an hemorrhage (arrow) in a larva of 14 days exposed to 20% of petroleum WSF; e – esophagus. 200x. Staining: H-E.

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

Figure 4. Liver from "pejerrey" *Odontesthes 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). 400x. 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, arrowheads), and also enlarged sinusoids (arrow). 400x. C. Liver from a larva of 21 days exposed to 10% of WSF, showing hypertrophy of the hepatocyte and its nucleum (arrow); s – sinusoid. 1000x. Staining: H-E.

Figure 5. Kidney sections of *Odontesthes 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). 400x. B. Anterior kidney of a larva of 21 days exposed by three weeks to 20% of petroleum WSF. An enlargement of glomerulus (arrow), intercellular spaces (arrowhead) and abnormal nucleus of epithelial cells. 400x. 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. 1000x. Staining: H-E.

Fig. 1

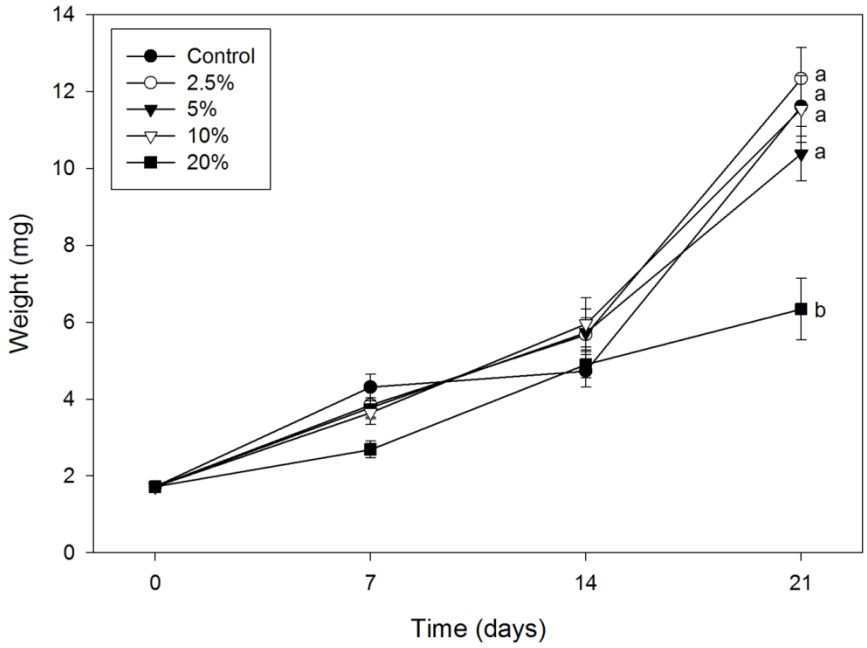

Control
2.5%
5%
10%
20%
Weight (mg)
Time (days)
0
2
4
6
8
10
12
14
0
7
14
21
a
a
a
a
b

Fig 2

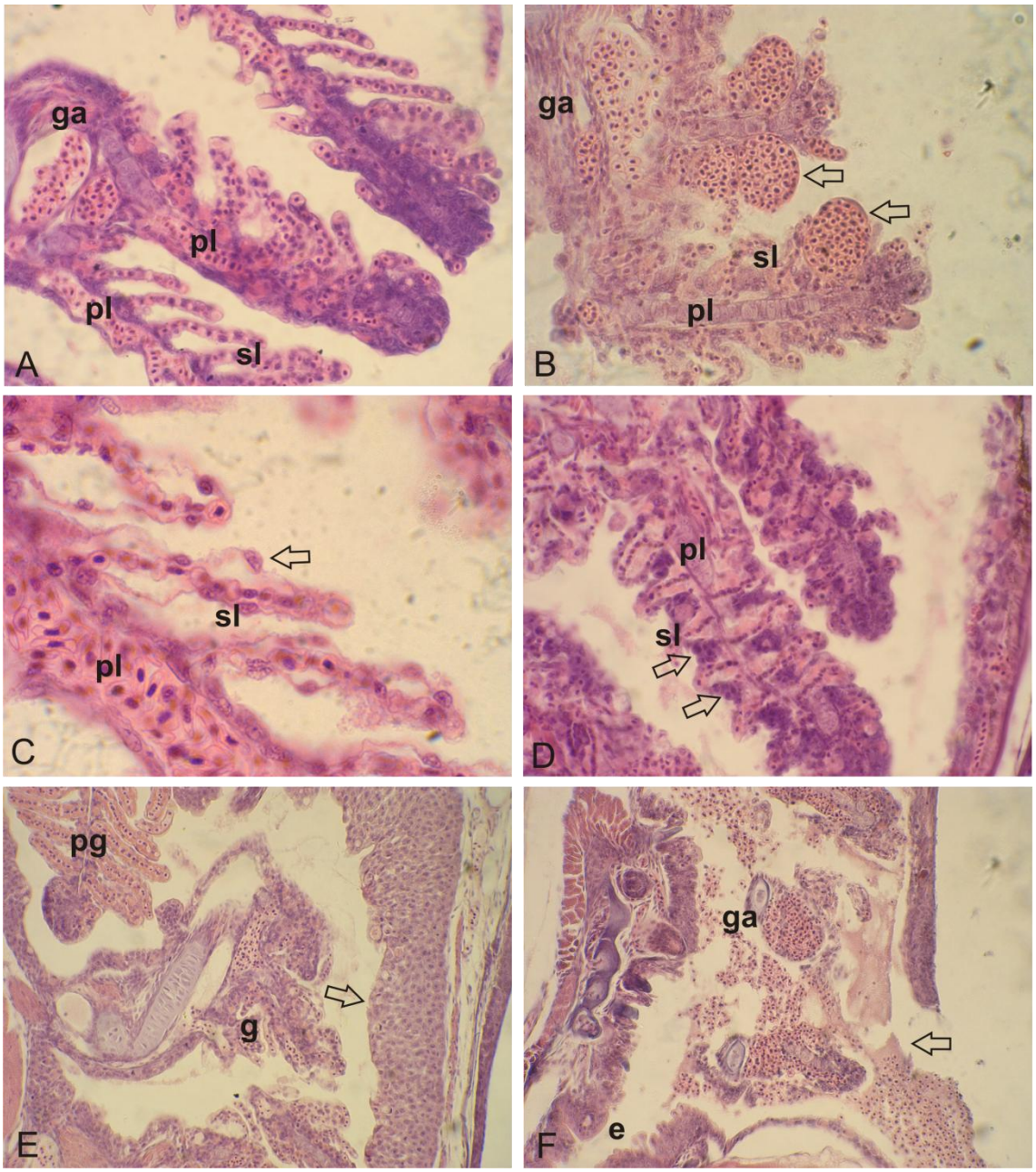


Fig 3

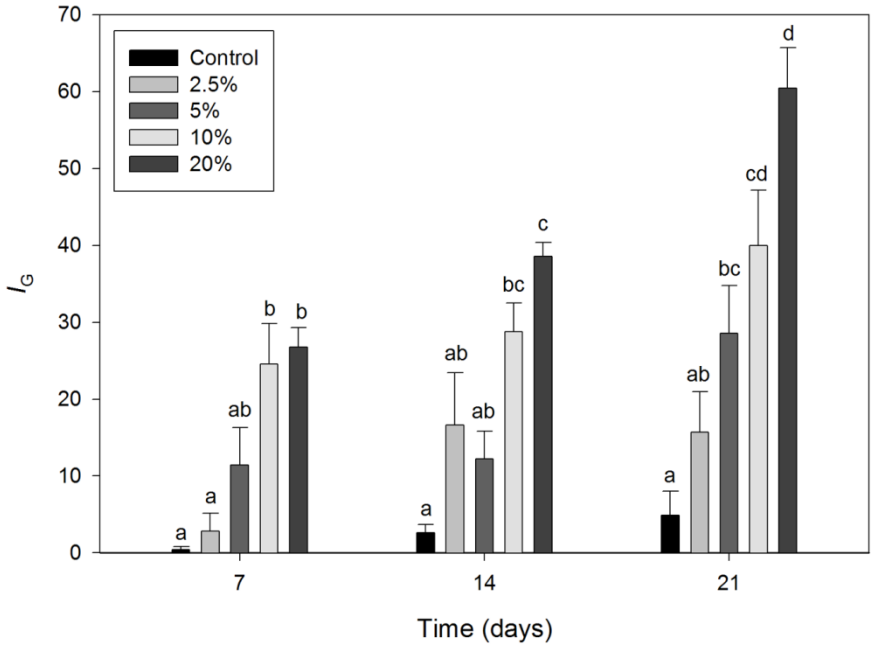

Control
2.5%
5%
10%
20%
I_G
70
60
50
40
30
20
10
0
a
a
ab
b
b
a
ab
ab
bc
c
a
ab
bc
cd
d
7
14
21
Time (days)

Fig 4

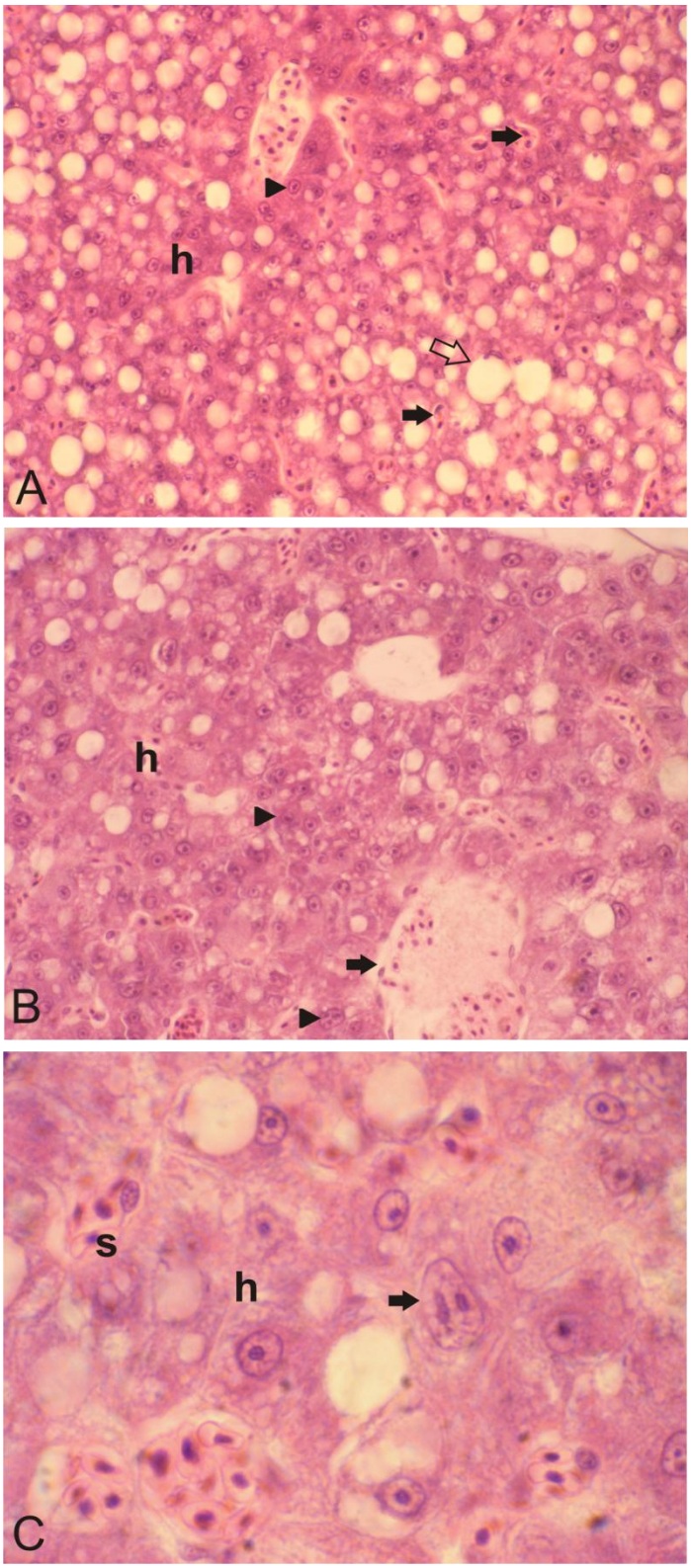
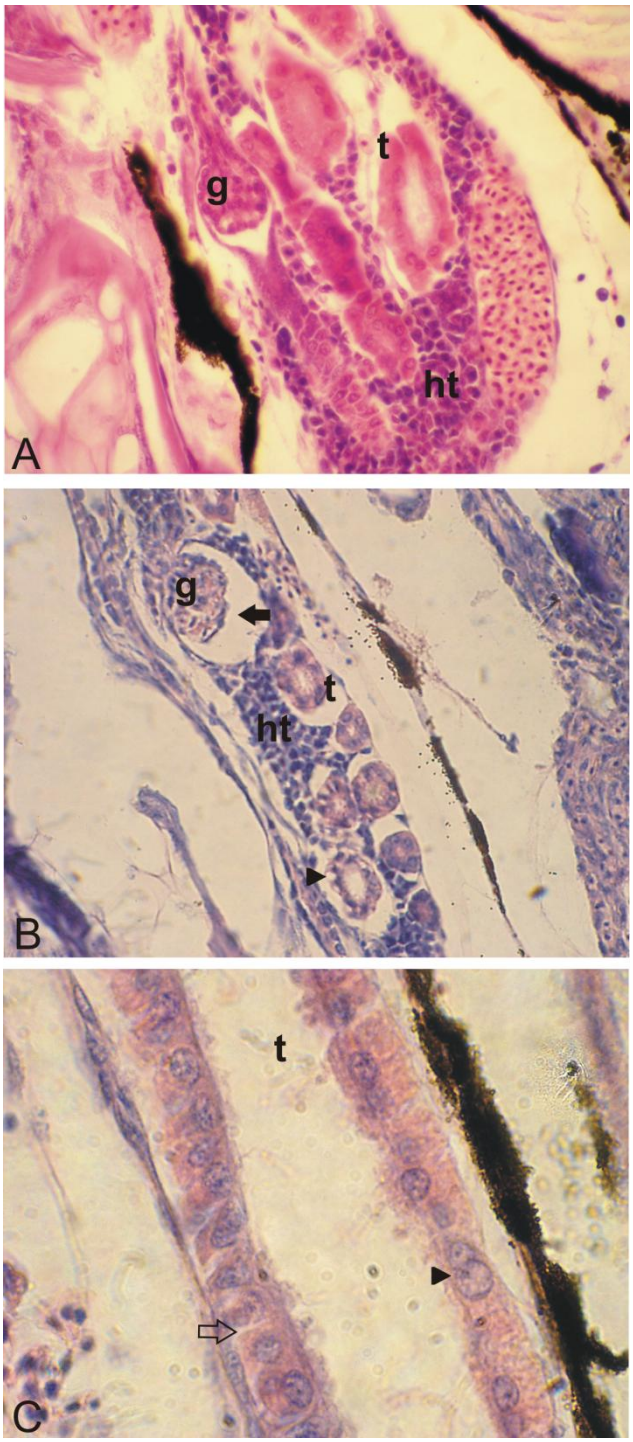


Fig 5



6. CONCLUSÕES

As larvas de peixe-rei sofrem danos irreversíveis quando expostas à FSA de petróleo. O nível de segurança e o fator de aplicação podem ser empregados para larvas do peixe-rei *O. argentinensis*. A histopatologia pode ser utilizada como biomarcador da exposição crônica de larvas do peixe-rei ao petróleo. O índice de lesão adaptado para a espécie reflete o grau de dano das brânquias expostas à FSA do petróleo. As larvas do peixe-rei *O. argentinensis* podem ser bioindicadoras ou biomonitoras da poluição em áreas costeiras.