I INTRODUÇÃO

O ambiente aquático é um dos ecossistemas que mais sofre impactos causados pela ação antrópica, uma vez que constitui o compartimento final de vários produtos gerados pela atividade humana (AKAISHI, 2003). Esses ecossistemas acabam refletindo com facilidade os efeitos de várias atividades que ocorrem ao seu redor, ou seja, estão expostos aguda e cronicamente a agentes químicos que são poluentes e que por sua vez prejudicam o desenvolvimento da biota. O comprometimento de processos fisiológicos vitais como respiração, reprodução e crescimento são exemplos das diversas perturbações metabólicas que os contaminantes ambientais podem causar aos organismos aquáticos (STEGEMAN *et al.*, 1992).

Vários fatores têm colaborado para o aumento significativo dos lançamentos de despejos e resíduos nos cursos d´água como o alto nível de industrialização, a intensa atividade agrícola, concentração das atividades humanas próximas de áreas onde se encontra a maioria dos recursos pesqueiros.

Um dos principais poluidores ambientais é o petróleo, sendo isso uma preocupação global. Nas últimas décadas têm havido um aumento da conscientização no que se refere aos riscos ambientais que envolvem as atividades industriais associadas à cadeia de produção de petróleo, no entanto, ainda são freqüentes os acidentes envolvendo esta substância.

1.1 Petróleo e a sua fração solúvel em água

O petróleo contribuiu para o desenvolvimento da humanidade, favorecendo o crescimento da indústria, surgimento de refinarias na produção de combustíveis e derivados. No entanto, junto com os benefícios para a humanidade muitos problemas surgiram e têm se agravado ao longo dos tempos. Dados estatísticos de agências de proteção ambiental dos países produtores de petróleo vêm demonstrando que as atividades relativas à exploração, refino, transporte e armazenamento de petróleo e seus derivados, têm um potencial de risco elevado, com grandes desastres ambientais em vários países nestas últimas décadas (TEAS *et al.,* 2001).

AKAISHI (2003) cita que, segundo o Conselho Nacional de Pesquisa dos Estados Unidos (1985), a exposição de organismos aquáticos ao petróleo e seus derivados pode potencialmente prejudicar os recursos pesqueiros de muitas maneiras, incluindo a redução nas taxas de estoque de peixes. A indústria petroquímica é uma das fontes mais poluidoras existentes, iniciando o ciclo de poluição desde o processo exploratório do petróleo até a sua distribuição final. Toda essa cadeia conjugada tem sido reportada nas últimas décadas com uma grande preocupação tanto do ponto de vista ambiental quanto energético (TANOBE, 2005).

O petróleo (óleo cru) no estado líquido é uma substância oleosa, inflamável, menos densa que a água, com cheiro característico e cor variando entre o negro e o castanho-claro (CEPETRO, 2006). É constituído por uma mistura de compostos orgânicos, sendo na sua grande maioria, 75%, por hidrocarbonetos, tanto de cadeias longas como de cadeias curtas (NEFF, 1978).

Segundo BRAUNER *et al.* (1999), os hidrocarbonetos de cadeias curtas são voláteis, permanecendo menos tempo nos ambientes aquáticos, no entanto são muitos mais tóxicos. Evaporação, dissolução, oxidação, sedimentação, biodegradação e absorção pela biota são os diferentes processos pelos quais o petróleo ou seus derivados passam após atingir o ambiente aquático. Tais processos determinam o destino destes produtos e os seus impactos sobre ambientes naturais. Geralmente a quantidade de óleo dissolvido na água é pequena, embora dependa da turbulência do corpo d´água. No entanto, é essa fração hidrossolúvel que causa os impactos mais imediatos aos organismos aquáticos, sendo assim considerado um importante determinante de toxicidade do petróleo e seus derivados em acidentes ambientais (SAEED; MUTAIRI, 1999).

ZIOLLI (1999) afirma que a fração do petróleo solúvel em água (FSA) é a principal responsável pelo impacto ambiental causado por compostos derivados de petróleo, tanto por ser visualmente imperceptível quanto pelas transformações químicas de seus constituintes iniciais.

A FSA e seus derivados são uma mistura complexa de hidrocarbonetos policíclicos aromáticos (HPA), fenóis e compostos heterocíclicos contendo nitrogênio e enxofre (ANDERSON *et al*, 1974; MACKAY; SHIU, 1976). Estudos indicam que a absorção da FSA por peixes teleósteos causa alterações que comprometem a sobrevivência desses

organismos no ambiente como danos estruturais nas lamelas respiratórias das brânquias de peixes (DIMICHELE; TAYLOR, 1978; POIRIER *et al*., 1989; CORREA; GARCIA, 1990; ENGELHARDT *et al.*, 1981; PRASAD, 1991), comprometendo as trocas gasosas com o meio e resultando em hipoxia, sendo a principal causa da morte acidental em massa; além disso, lesões hiperplásicas envolvendo células mucosas foram observadas (SPIES *et al*., 1996).

Sendo assim, inúmeros estudos demonstram que vários componentes do petróleo são capazes de causar danos das mais diferentes naturezas, como alterações no comportamento reprodutivo e alimentar, danos cromossomais, aberrações celulares, entre outros.

1.2 Embriotoxicidade

É muito importante o conhecimento da toxicidade de agentes químicos no meio hídrico, além do estabelecimento de limites permissíveis de várias substâncias químicas para a proteção da vida aquática, a determinação da toxicidade de agentes químicos serve para avaliar o impacto momentâneo que esses poluentes causam à biota dos corpos hídricos (BERTOLETTI, 2006). O embrião em desenvolvimento é geralmente considerado o estágio mais sensível do ciclo de vida de um peixe (HALLARE *et al.,* 2006). Estudos prévios revelam que a sensibilidade de embriões e larvas a agentes químicos é muito maior que para adultos (LUCKENBACH *et al.,* 2001; EATON *et al.,* 1978; McKIM, 1977; ROSENTHAL; ALDERDICE, 1976; SKIDMORE, 1965). Muitos desses poluentes podem ter tóxicos aos embriões, ocasionando alterações em processos fisiológicos, mal formações e até mesmo, genotoxicidade.

Devido a sua imobilidade os embriões são mais afetados que os adultos pelos agentes tóxicos ambientais e devido a sua imaturidade fisiológica possuem baixos níveis de enzimas necessárias para a desintoxicação. Além disso, estão apresentando um gasto energético muito grande para sua formação e a exposição a poluentes demanda um gasto extra de energia para a biotransformação dos mesmos.

Neste trabalho foram estudados os efeitos tóxicos que a FSA pode desencadear aos embriões de *Danio rerio* através da avaliação de algumas variáveis como mortalidade, freqüência de batimentos cardíacos, defeitos na cauda e nos olhos e pigmentação.

1.3 Biotransformação

A biotransformação, ou seja, a transformação metabólica de compostos químicos é necessária para que haja a alteração da atividade biológica do composto e para que a interação entre a célula afetada e o agente agressor cesse. O processo de biotransformação tem a função de converter estruturas tóxicas para menos tóxicas e excretar rapidamente convertendo químicos lipofílicos em estruturas hidrofílicas. O metabolismo das drogas envolve dois tipos de reação bioquímica, conhecidas como reações de fase I e de fase II. Freqüentemente essas reações ocorrem seqüencialmente, mas isso pode variar (RANG; DALE, 2003).

As reações de fase I introduzem ou expõem um grupo funcional no composto original através de reações oxidativas (desalquilação, hidroxilação, oxidação e desaminação) e reações de hidrólise. Geralmente, a conversão metabólica de compostos químicos tem natureza enzimática.

As enzimas do citocromo P450 são importantes catalisadores de processos de biotransformação, através de reações oxidantes e redutoras exercendo atividade contra um grupo de substratos quimicamente diferentes. Em peixes, e em outros vertebrados, o citocromo P450 é principalmente encontrado no retículo endoplasmático (RE) e nas mitocôndrias de fígado, rim, cérebro, e intestino delgado, assim como em outros órgãos (BUCHELI; FENT, 1995).

Uma resposta sensível à exposição de animais a hidrocarbonetos policíclicos aromáticos, bifenilas policloradas e dibenzodioxinas é a indução de isoenzimas específicas do citocromo P450. Ocorre então a transcrição do gene para CYP1A, mediada por estimulação do receptor, resultando no aumento do nível de RNA mensageiro especificando nova síntese de isoenzimas de citocromo P450 (CYP1A) e no aumento da respectiva atividade catalítica, ou seja, atividade da etoxiresorufina-*O*-deetilase (EROD) (STEGEMAN; HAHN, 1994).

Estudos têm mostrado que há uma relação concentração dependente entre o aumento do conteúdo enzimático e a atividade induzida do CYP1A em peixes quando expostos a hidrocarbonetos policíclicos aromáticos (GUENGERICH; MACDONALD, 1990; GOKSΦYR; FÖRLIN, 1992; STEGEMAN; HAHN, 1994; BUCHELI; FENT, 1995; DIGIULIO *et al.,* 1995).

A EROD catalisa uma reação de O-desalquilação, dependente de NADPH, na qual o substrato é a 7-etoxiresofurina (7ER) (STEGEMAN; HAHN, 1994), portanto a atividade catalítica do CYP450 pode ser avaliada pela atividade desta enzima. O aumento da atividade da EROD em vertebrados é um indicador da indução do CYP1A, auxiliando, portanto, no monitoramento ambiental (BUCHELI; FENT, 1995).

As reações de fase II de biotransformação são reações de conjugação, isto é, há fixação de um grupo substituinte. O conjugado resultante é quase sempre inativo e menos lipossolúvel do que seu precursor, sendo excretado na urina ou na bile. As reações que ocorrem nesta fase são reações de glicuronidação, sulfatação e acetilação (GOODMAN;GILMAN, 1996).

Glutationa transferases, UDP-glucuronosiltransferases e sulfotrasnferases são as enzimas mais estudadas da Fase II, sendo utilizadas como biomarcadores de efeito ou de exposição, uma vez que são alteradas por vários xenobiontes (HUGGETT *et al*., 1992).

A glutationa S-transferase (GST) atua na biosíntese de metabólitos do ácido araquidônico (leucotrienos e prostaglandinas), na isomerização de esteróides, no transporte intercelular de compostos endógenos como heme, bilirrubina, hormônios esteróides e participa da conjugação da glutationa (GSH) catalisando a conjugação como primeiro passo na formação de metabólitos para excreção, como o ácido mercaptúrico (MALLINS; OSTRANDER, 1993).

MALLINS e OSTRANDER (1993) afirmam ainda que a grande maioria das GSTs realizam a conjugação através de um substrato artificial, o 1-cloro-2,4-dinitrobenzeno (CDNB). A GST é uma enzima bastante comum em várias espécies, tendo sido identificada em procariontes, leveduras, plantas, moluscos, crustáceos, insetos, peixe, anfíbios e mamíferos, representando o maior grupo de enzimas desintoxicantes. Sua estimulação envolve reações de conjugação na presença de glutationa (Da SILVA, 2004).

Animais aquáticos que habitam ambientes poluídos podem estar expostos a xenobióticos, os quais sofrem desintoxicação mediada pela glutationa na sua forma reduzida, catalisada pela enzima glutationa S-transferase. Esta enzima de biotransformação tem sido estudada em trabalhos de campo no monitoramento de poluentes de origem industrial (CHO *et al.,* 1999).

1.4 *Danio rerio*

Para a realização dos experimentos foi escolhida a espécie de peixe *Danio rerio* (Hamilton*,* 1822), conhecido como peixe zebra, ou paulistinha. É um peixe ciprinídeo (família Cyprinidae) com padrão de coloração característico, com listras pretas que o fazem semelhante a uma zebra. Peixe originário da Ásia: Paquistão, Índia, Bangladesh, Nepal e Myanmar.

É uma espécie facilmente mantida em condições controladas de laboratório (temperatura da água 25,0 ± 0,5^{o}C, pH 7,0 ± 0,2 e fotoperíodo de 12h claro/12h escuro), não requer muitos cuidados para sua criação e é facilmente encontrado em lojas comerciais. Os adultos são nadadores rápidos, que chegam ao comprimento de 4 a 5 centímetros. Alcançam maturidade sexual com 10 a 12 semanas, e o pico de desova ocorre de 5 a 10 dias – cada fêmea produzindo, em média, 150 a 400 ovos por dia. Os ovos, transparentes e pequenos, são fertilizados externamente. Também têm a característica de não serem adesivos. A eclosão dos ovos se dá entre 48 e 96 horas (WESTERFIELD, 2000). O embrião do peixe zebra é transparente nos primeiros estágios de desenvolvimento permitindo fácil identificação, estudo das estruturas neurais e observação de más formações. Tal transparência é ideal para localização imunohistoquímica e para técnicas de marcação de proteínas. Os embriões passam por um rápido desenvolvimento, com aparecimento de neurônios dentro de 24 horas após a fertilização (SCALZO; LEVIN, 2004).

O peixe zebra vem sendo utilizado há mais de 30 anos para estudar processos de desenvolvimento embrionário e algumas doenças**.** Apresenta o sistema nervoso central relativamente simples, comparado com roedores e por isso pode ser utilizado em pesquisas de comportamento, controle motor, aprendizado, memória e interações sociais. É uma

espécie de peixe com rápido crescimento sendo possível estudar a maioria dos órgãos nos primeiros dias de vida do peixe (GOLDSMITH, 2004).

Segundo HALLARE *et al.* (2005), ensaios com ovos do peixe zebra têm ganhado evidência em estudos ecotoxicológicos nesses últimos anos. Na Alemanha, por exemplo, o *DarT* - teste com embriões do peixe zebra, como foi denominado por NAGEL (2002) – tem sido validado para uso em testes com agentes químicos e avaliação de efluentes.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar a toxicidade ao embrião e a biotransformação em peixes juvenis da espécie *Danio rerio* expostos à fração do petróleo solúvel em água.

2.2 Objetivos específicos

- Determinar os efeitos embriotóxicos da fração do petróleo solúvel em água em *Danio rerio.*

- Avaliar a biotransformação da fração do petróleo solúvel em água através da atividade da etoxiresorufina – *O* – deetilase (EROD), reação de fase I e da Glutationa S-transferase (GST), reação de fase II, da biotransformação em peixes juvenis da espécie *Danio rerio*.

Evaluation of Embryotoxic Effects and Biotransformation of Water Soluble Fraction in Zebrafish (*Danio rerio,* Hamilton, 1822)

1 Introduction

The oil contributed for the development of the humanity, favoring the growth of the industry, sprouting of refineries in the fuel production and derivatives. However, together with the human benefits lots of problems have arisen and it's getting worst during the years. Statistical data of environmental protection agencies about countries that produce oil have been demonstrating that the relative activities to the exploration, refining, transport and storage of oil and its derivates, have a high risk potential related with environmental disasters in these last decades (TEAS *et al.,* 2001).

Evaporation, dissolution, oxidation, sedimentation, biodegradation and absorption by the biota are the different processes that the oil or its derivatives goes through to reach the aquatic environment. Such processes determine the destination of these products and its impacts on natural environments. Generally, the amount of oil dissolved in the water is low; however, it is the soluble fraction that causes serious impacts to the aquatic organism (SAEED; MUTAIRI, 1999). Many studies have indicated that water soluble fraction (WSF) of crude oil is a complex mixture that contains polycyclic aromatics hydrocarbons (PAHs), phenols and heterocyclic compounds containing sulphur and nitrogen (ANDERSON *et al*., 1974; MACKAY; SHIU, 1976). Studies indicate that the absorption of the WSF by teleosts fish cause alteration that compromises the survival of these organisms in the environment. The hydrocarbons derived from the oil provoke structural damages in respiratory gills lamellas of fish. Studies show that components of the oil are capable to cause alterations in the reproductive and nutritional behavior, genetics damages and cellular aberrations, among others. For all these reasons it is important to study the effects of the water solution fraction (WSF) of crude oil in the aquatic organisms.

The biotransformation of chemical compounds in the organism is essential to modify the biological activity of the toxic agent, with intention to convert lipophilics chemistries into hydrophilic structures to be quickly eliminated (GILMAN, 1996). The biotransformation includes numerous enzymatic systems, which act in differents types of substrates.

Cytochrome P4501A (CYP1A) is a component of phase I of detoxification pathway of organic chemicals such as polycyclic aromatic hydrocarbons (LIVINGSTONE, 1991)

that can be oxidatively metabolized by induction of 7-ethoxyresorufin- *O*- deetilase (EROD) (EGGENS; GALGANI, 1992). So the cytochrome P4501A enzyme system function can be measured using EROD activity as a biomarker. This enzyme catalyzes an O-dealkylation, dependent of NADPH, in which one the substrate is 7- ethoxyresorufin (7ER). The induction of this enzyme activity is a sensitive parameter of exposure to some xenobiotics compounds, such as PAHs (STEGEMAN; HAHN, 1994).

Glutathione S- transferase (GST) is a common enzyme in some species, having been identified in plants, yeast, mussel, crustaceans, insects, fishes, amphibians and mammals. Its stimulation involves reactions of conjugation (phase II of detoxification) in the presence of glutathione (MALLINS; OSTRANDER, 1993). Aquatic animals that inhabit polluted environments can be exposed to xenobiotics, which are detoxificated by glutathione in its reduced form, catalyzed by the enzyme glutathione S-transferase. This biotransformation enzyme has been studied in monitoring programs (CHO *et al*., 1999).

According to OBEREMM (2000), for many years researchers all over the world have used fish embryo test to evaluate chemical effects. The researchers found out that embryo assays are much more effective when compared to short-term tests using juvenile and adult fish. Other advantage in using embryos is that they offer much more diverse endpoints for evaluation effects than the use of juveniles and adult fish.

Due to theirs immobility, the embryos are more affected than the adults by toxic agents. According to HALLARE *et al.* (2005), studies with zebrafish´s eggs have gained evidence in the last years and become a tool used to chemicals compounds and to determine the maximum allowable concentrations (MAC) of solvents to be used in experimental systems. In Germany, for example, the *DarT* – test with embryos of zebrafish, as was denominated by NAGEL (2002) – has been used to evaluate wastewaters and chemical agents.

The zebrafish (*Danio rerio*) has been widely used, mainly, to study embryonic development and some diseases. It is a small freshwater fish that reproduces all over the year and the eggs are transparent. *Danio rerio* presents some advantages as test model, such as a short generation time, high fecundity and rapid development; besides, external fertilization and translucent embryos (OBEREMM, 2000; WESTERFIELD, 2005).

In recent decades, the development of industrial and urban centres has increased the levels of petrochemical products in the environment (LIMA *et al.,* 2006) besides this, they provoke irreparable damages to aquatic ecosystems and due to the importance of dissolution processes following oil spills the aims of the present study were to evaluate the embryotoxicity of the WSF of crude oil and the biotransformation through the EROD and GST activities, in juveniles zebrafish. The embryos and the juveniles were sensitive and seriously affected to oil spill.

2 Materials and methods

2.1 Maintenance of fish

Sexually mature zebrafish (*Danio rerio*) were maintained in a 15 liters aquaria at temperature 25,0 ± 0,5^{o}C, pH 7,0 ± 0,2, and 12h light/ 12h dark photoperiod. They were fed twice daily with commercially available artificial diet (Tetramin flakes). In the aquaria, were put a grid to avoid the fishes to eat the eggs. Based on WESTERFIELD (2005), six fish per aquaria were used in a ratio of 1 male to 2 females.

2.2 Test solutions

The crude oil was obtained from Campus Bay (Petrobras Company) and the WSF was prepared according to ANDERSON *et al.* (1974), by placing 1 part oil over 9 parts of reconstituted water (0,1335g/L $MgSO_4$; 0,0004g/L KCl; 0,0065g/L $CaCl_2$; 0,0105g/L $NaHCO_3$; pH 7,2-7,3) in a Pyrex bottle and slowly stirring the water for a period of 20h, 20,0 ± 2,0^{o}C. To avoid the evaporation of the volatile hydrocarbons the bottle was capped with a plastic foil and a black plastic was used to avoid the light interference. After the mixture, the oil and the water soluble part were separated. This solution was considered the 100% soluble fraction. From this solution the 50%, 33% and 15% solution were prepared.

A chemical analysis (total petroleum hydrocarbons – TPH – and oil and grease) of the 100% fraction was carried out in LACTEC laboratory – Technology Institute, Paraná Federal University based on Standard Methods for the Examination of Water and Wastewater SM 5520F and SM 5520 B, in order to confirm the presence of hydrocarbons.

As a positive control, a solution of ethanol 2% was used, it was obtained from 99,9% ethanol and distillated water.

2.3 Zebrafish embryo test

The eggs were collected in the morning, rinsed several times with distillated water and immediately transferred to chambers containing different concentrations of WSF of

crude oil (15%, 33% and 50%), 2,0% ethanol as positive control and reconstituted water as control to observe the embryo development. In each chamber was put 10 eggs, and for each WSF, ethanol and reconstituted water was used 9 chambers. The experiment was realized twice, totalizing 180 eggs for each group. To avoid the evaporation all the chambers were covered and the media were replaced every 24h. The incubation temperature was 28,5^{o}C. The development of embryos was monitored at 2-4, 24, 48, 72 and 96h after fertilization.

During the observation, dead embryos were removed to avoid contamination of the surviving ones. Data about embryo mortality, tail and eyes defects, pigmentation and heartbeat were observed in each time using a microscope. The no exposed group presented approximately 180 beats per minute. So, in this study was considered a reduced heartbeat less than 60 beats per minute.

2.4 Enzymatic assays

For GST and EROD activities analyses juveniles zebrafish were exposed in a 15 liters aquaria to reconstituted water and WSF in three different concentration 15%, 33% and 50% (n=34). The fishes were not fed during the exposition. After 96 hours the fishes were sacrified, the head and tail were cut and the bodies (pool of 2 animals) were storage in – 70^{o} C. The S_9 fraction was obtained after the sample homogenization with 2mL of phosphate buffer (pH 6,5) using Potter-Elvejhem, and centrifugation for 30 minutes, 10.000 x g at 4^{o}C, based on the methodologies described by STEGEMAN, BINDER and ORREN (1979) and SILVA DE ASSIS (1998). The S9 fraction was aliquoted to the EROD and GST activities analysis. The EROD activity was assayed spectrofluorimetrically using 7-etoxyresorufin (7 ER) as substrate (2,6 μM in Tris-NaCl buffer 0,1 M pH 7,5) and expressed in pmol.min^{-1}.mgprotein^{-1}. The sample and the 7ER buffer were incubated for 5 minutes at 27^{o} C. After this, 30 μL of NADPH (2,6mM) was added, and the measure was carried out in a Shimadzu spectrofluorimeter using 530nm and 590nm, excitation and extinction wave length respectively.

GST activity was measured with 1-chloro – 2,4 – dinitrobenzene (CDNB) (3mM) as substrate and glutathione (GSH) (3mM). The enzymatic determination was performed in microplate reader Sunrise – TECAN (wave length 340nm) and was expressed in nmol.min^{-}

^{1}.mgprotein^{-1}. The protein concentration was measured spectrofotometrically at 595 nm by the Bradford method (1976) using bovine serum albumin as standard.

2.5 Statistical analysis

The normality of the data was tested. One-Way Analysis of Variance (ANOVA) followed by Bonferroni´s test was performed to test the differences between control and treated groups. Differences were considered significant when $p < 0,05$. The enzymatic data were expressed as mean ± standard error (n = 17).

The embryotoxicity data were expressed in percentage based on HALLARE *et al.* (2006).

3 Results

3.1 Chemical analysis of WSF of crude oil

The WSF of crude oil considered the 100% concentration presented 442,5mg/L of TPH (Total Petroleum Hydrocarbons) and 1039,6mg/L of oil and grease. This result confirmed the presence of petroleum hydrocarbons in high concentration.

3.2 Embryotoxic effects

In the present study, was determined the embryotoxicity of WSF of crude oil in three different concentration (15%, 33% and 50%) and ethanol as positive control. All of these groups were compared to a no exposed group.

The developmental stages of the no exposed group (control group) was normal, as described in the literature. Around four hours of development was formed a elevated cap of regular small cells on top of the yolk and at 24 hours was observed the basic vertebrate body organization, beginning of heartbeat and spontaneous movements. The completion of rapid morphogenesis of primary organ systems, cartilage development in head and pectoral fin occurred at 48 hours. The next stages observed were the hatching process, blood

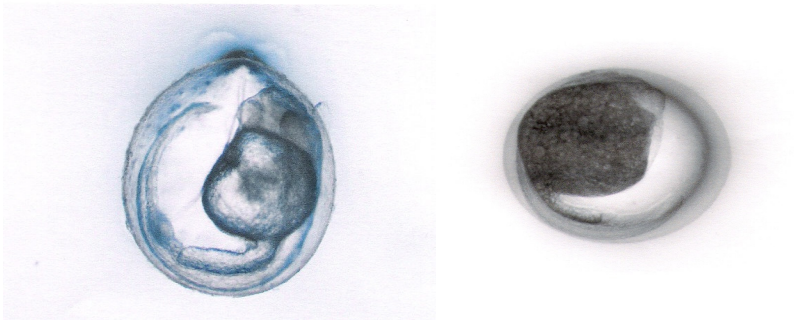
circulatory system fully developed, swim bladder inflates, swimming and feeding. The control group showed a normal embryonic development with the formation of all the structures (Figure 1A, 2B and 2C). The normal embryos presented a well-developed head, tail region and body. Spontaneous movements started at 24 hours of development and it was possible to notice the black pigmentation of the embryos macroscopically. The hatching was normally during the experiment and the 96 hours survival rate was high. The no exposed embryos presented 180 a 200 beats per minute.

The ethanol-treated embryos showed different toxic effects and this substance showed to be a good positive control (Table 1). The hatching process was delayed when compared to no exposed embryos, in some cases a total absence of hatching. No eyes defects were observed. After 96 hours of exposure many embryos presented weak or no pigmentation. The heartbeat was reduced (< 60 bpm) in 64% of the analyzed embryos. During the microscopic observations it was possible to notice that the embryos were smaller than the no exposed one and that no spontaneous movements happened.

The worst effects were observed in WSF of crude oil embryos-exposed (Table 1). In the three concentrations (15%, 33% and 50%) a significant embryos numbers died during the exposure. Tail defect was not a pronounced effect and occurred in a lower percentage when compared to ethanol-exposed embryos. No eyes defects were observed. The heartbeat was reduced in all three concentrations. There was no difference between 33 and 50% concentration in the heartbeat. The pigmentation was a concentration parameter because at 15% concentration 64% of the embryos presented weak pigmentation, and to 33 and 50% the percentage was 89 and 92%, respectively. During the observation the embryos exposed to WSF of crude oil were not responsive to stimulations and like the ethanol-exposed embryos no spontaneous movements happened.

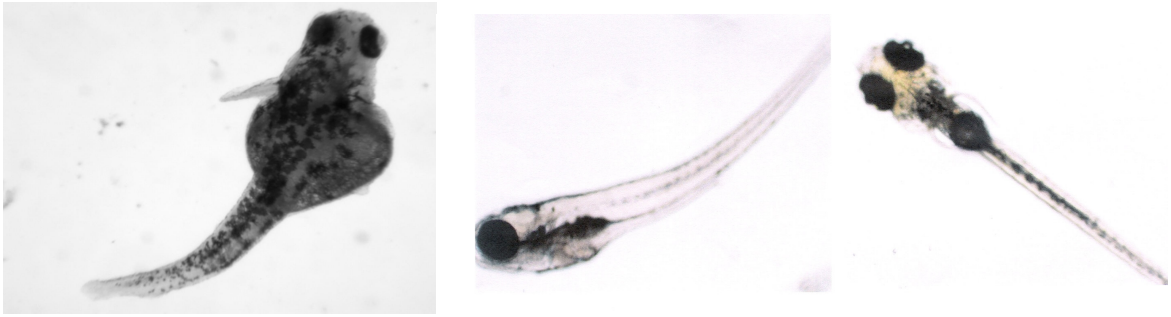
Table 1. Percentage of variation of parameters quantified in zebrafish embryos (n=180) exposed to water soluble fraction (WSF) of crude oil for 96 hours.

Development defects	**Control**	**WSF 15%**	**WSF 33%**	**WSF 50%**	**Ethanol 2%**
Tail defects	0	11	17	20	25
Reduced heartbeat	0	90	93	93	64
Weak pigmentation	0	64	89	92	34
Eye defect	0	0	0	0	0
Mortality	22	94	100	100	89



A B

Figure 1. Embryonic development of *Danio rerio* in 24 hours. A: a normal embryo (24h) with well developed head, eyes and tail. B: an embryo (24 h) with no head, tail defects exposition to ethanol 2%.



A B C

Figure. 2. Embryonic development of *Danio rerio* in 72 hours. A: 72 hours embryo with abnormal body structure and tail defect exposed to WSF crude oil (33%). B and C: control embryo with well-developed structure (72 hours), in lateral and dorsal view, respectively.

3.3 Enzymatic analysis

No alteration was observed in the EROD activity of the exposed group compared to control (data not shown).

The activity of GST in the fish exposed to 33% and 50% increased significantly compared to control group ($p < 0,001$ and $p<0,05$, respectively). Although, the activity of GST at 15% compared to control was not increased ($p>0,05$). There was no significant difference between 33% and 50%, but between 15% and 33% ($p<0,05$) (Figure 3).

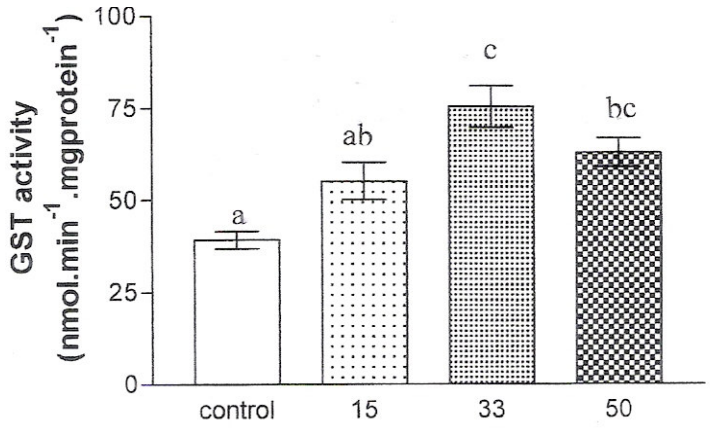


Figure 3 – Glutathione S-Transferase activity in *Danio rerio* exposed to different concentrations of water soluble fraction of crude oil (15%, 33% and 50% and control). Different letters indicate significant differences among treatments.

4 Discussion

The zebrafish early life stage test has become a tool widely used to evaluate toxic effects of chemical and wastewater (HALLARE *et al*., 2004; NAGEL, 2002; LANGE *et al*., 1995; SCHULTE; NAGEL, 1994) due to offer information for the short-term detection of chemically mediated aquatic effects (OBEREMM, 2000) and because its embryonic development is well characterized and readily visualized (BRADFIELD, 2006). In the present study, there were determined the embryotoxicity of water soluble fraction of crude

oil in three different concentrations (15%, 33% and 50%) and of ethanol used as positive control. A test model of ethanol using zebrafish embryos was used by BRADFIELD *et al.* (2006) and also induced embryotoxicity. They showed that the embryonic zebrafish model has several advantages over mammals including high yield of synchronously fertilized eggs, transparency of embryos and rapid embryonic development. LOUCKS *et al.* (2004) showed alterations in neurocranial and craniofacial skeletal development and growth retardation when exposed zebrafish embryos to ethanol, and he related its effects to those in human.

WIEGAND *et al.* (2001) and HALLARE *et al.* (2005), observed in *Danio rerio* embryotoxic effects as reduced heartbeat, alterations in movements and in the circulatory system, deformations and differences in pigmentation when exposed to atrazina and solvents as ethanol and dimethyl sulfoxide (DMSO). Our results clearly supported the HALLARE *et al.* (2005) study on the effects of ethanol in zebrafish embryos.

The concentrations of WSF of crude oil used in this study tried to reproduce a real condition of an accident involving crude oil. In an oil accident different processes in water occur and change the oil characteristics, and its products sometimes, become more toxic. During the dispersion the oil spot is broken in smalls spots increasing the surface contact with water. The dissolution and dispersion of the WSF compounds are important chemical process because their products remain in water even when the oil spot is removed. The chemical analysis of the WSF of crude oil in this study showed a high concentration of petroleum hydrocarbons, products that cause serious injuries. The embryos exposed to the three concentrations presented some alterations as tails defects and a high delay on hatching process that could interfere in the normal development.

The reduced pigmentation is a sensitive parameter and it was observed for many authors with different xenobiotics. The WSF exposure caused also a weak pigmentation corroborating with the results of others works. (HALLARE *et al*., 2005; SCHULTE, 1997).

HALLARE *et al.* (2005) exposed zebrafish embryos to organic sediments (heavy metals, PAHs – perylene) and showed significant developmental defects, as in our study. Other xenobiotics as methylmercury, DMSO and acetone also caused toxicity in *Danio rerio* embryos (SAMSON; SHENKER, 2000; HALLARE *et al*., 2005).

The EROD activity is known to be inducible by PAHs, but some studies show that EROD activity responds in different ways to some xenobiotics. REGOLI *et al.* (2002) studied for three years the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet, *Mullus barbatus,* exposed to dredged materials (containing PAHs and organic compounds) in the Mediterranean, and observed differences to EROD activity. In the first year, there was no variation in EROD activity, in the second year there was an induction of EROD activity and in the last year, again, no induction in this enzyme activity was obtained. In our study, no alterations in EROD activity was observed. One explanation for this result is the exposure time, maybe not sufficient to induce the enzyme. It is known that is necessary a gene transcription to activation the aryl hydrocarbon receptor (AHR) pathway tissue-specifically to induce distinct patterns of CYP1A expression. The aryl hydrocarbon receptor controls a battery of genes involved in PAH metabolism, such as cytochrome P4501A (INCARDONA *et al*., 2006). The other explanation was the tissue used to measure CYP1A induction. In this study was used the hole body fish to study CYP1A induction. It is known that the liver is the major metabolic organ in fish, but in this study was not possible to use only the liver, because of the small size of such organ. ORTIZ-DELGADO *et al. (*2006) compared xenobiotic CYP1A induction in liver, gills and excretory kidney of gilthead seabream, *Sparus aurata* exposed to benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The magnitude of the inducted response varied with the organs and with the chemical compounds.

Glutathione S-transferase catalyzes the conjugation of eletrophilic xenobiotics to glutathione (GSH) (GADAGBUI; JAMES, 2000) and plays an important role in protecting tissues from oxidative stress (FOURNIER *et al*., 1992). JIFA *et al.*(2005) affirm that GST responds differently to different compounds exposure. In the present study, the GST activity in the juveniles fish was induced to 33% and 50% fraction. It is possible to affirm that the GST was involved in elimination of the WSF of crude oil once this enzyme is related to detoxification process. In goldfish *Carassius auratus* exposed to the water soluble fraction of diesel oil, ZHANG *et al.* (2004) observed an increasing at GST activity in high concentration of PAH compared to low concentration. Probably, in the fish exposed to the low concentration of PAH, occurs an inappropriate conjugation and consequently an accumulation of metabolites oxygen reactive causing cellular injury. GST was also induced

in other organs than the liver. Extrahepatic GST activity has been demonstrated in a number of species, the other organs involved are the intestine and gills (MALLINS; OSTRANDER, 1993).

In field studies, CHEUNG *et al.* (2001) showed a increase in GST activity in the digestive gland of the mussel *Perna virides* and GOWLAND *et al.* (2002) observed the same result when exposed *Mytilus edulis* at high molecular weight PAHs. ZACCARON *et al.* (2005) observed an increasing of GST activity in oysters *(Crassostrea rhizophorae)* exposed to diesel oil.

Danio rerio showed to be a good option to study effects of toxic agents, the WSF was embryotoxic to zebrafish and altered the biotransformation enzyme GST. However, further enzymatic studies are necessary considering activity in different tissues and time of exposure.

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3 CONSIDERAÇÕES FINAIS

O petróleo proporcionou um desenvolvimento importante para a civilização, no entanto, sua exploração tem trazido danos irreparáveis ao meio ambiente. Esses acidentes ocorrem freqüentemente no mundo todo. No Brasil, praticamente todos os anos algum acidente ocorre e inúmeros litros de petróleo são despejados na natureza (AMBIENTE BRASIL, 2006). Estima-se que, no total, esses grandes derramamentos tenham sido responsáveis por um volume em torno de 3,9 bilhões de litros de óleo despejados, principalmente em ambiente marinho (AMBIENTE BRASIL, 2004).

Quando um acidente com petróleo ocorre, muitos processos químicos (evaporação, dissolução, dispersão) acontecem e a ciência ainda não conseguiu mensurar todos os prejuízos que um acidente desse tipo causam ao meio ambiente. Sabe-se que os prejuízos imediatos são grandes; no entanto, ainda não se conhece os efeitos em longo prazo. Mesmo depois da mancha de óleo ser removida, muitos outros componentes altamente tóxicos continuam em contato com a água e com os organismos do ecossistema afetado.

Além dos animais, os compostos tóxicos presentes no petróleo afetam também as populações que dependem dos estoques pesqueiros dessas áreas. Como foi demonstrado nesse estudo, a fração hidrossolúvel do petróleo altera o desenvolvimento embrionário normal dos peixes, o que acarretará em diminuição das espécies presentes em uma área afetada pelo derramamento de petróleo. É importante lembrar que há outras formas de contaminação além dos acidentes. Por ordem de importância pode-se citar as águas de lavagens dos tanques dos petroleiros, as águas de lastro (sistema utilizado para manter a estabilidade do navio) e efluentes de praças de máquinas dos navios, os despejos de refinarias costeiras, a operação de petroleiros e outros tipos de navios, além de efluentes industriais e municipais contaminados por óleo e pequenas contribuições de exsudações naturais (BARCELLOS, 1986). Desta maneira, os organismos aquáticos, principalmente os que habitam regiões costeiras, sofrem impacto constante por hidrocarbonetos, sendo de grande importância seu monitoramento.

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