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Embryotoxicity and Biotransformation Responses in Zebrafish Exposed to Water-Soluble Fraction of Crude Oil

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Abstract The toxic effects of water-soluble fraction (WSF) of crude oil (API27, Petrobras Campos Basin, Brazil) were evaluated during the early life stages of zebrafish, as well as its biotransformation in juvenile fish. Embryonic development was studied during 96 h. Reduced heartbeat rate, weak pigmentation, tail defects, and embryo mortality were observed for all of the tested concentrations of the WSF. Activities of the biotransformation enzymes were induced at the highest concentrations, showing that these enzymes played a role in its elimination. As shown in this study the crude oil WSF altered the normal embryonic development of fish.

Keywords *Danio rerio* · Zebrafish · Crude oil · Embryotoxicity · Biotransformation

Due to increased urban and industrial activities, water bodies are contaminated with different types of chemicals, such as polynuclear aromatic hydrocarbons (PAHs), which affect the inhabiting aquatic organisms and thereby potentially harm the ecosystem. Environmental accidents, such as oil spills, aggravate this problem, and biomonitoring studies using new methods to evaluate the detrimental effects on biota have been increasing in number over the past few decades (Silva et al. 2009). Crude oil is highly unstable in the aquatic environment, quickly loosing the volatile fractions through weathering. The water-soluble fraction (WSF) of crude oil and their derivatives products contains a mixture of polycyclic aromatic hydrocarbons, monoaromatic hydrocarbons often referred to as BTEX (benzene, toluene, ethylbenzene and xylene), phenols and heterocyclic compounds, containing nitrogen and sulfur and also heavy metals. The deleterious effects of petroleum are the result of physical fouling and the intake of water-soluble and insoluble hydrocarbons by aquatic biota (NAP 2003). In addition, the absorption of the WSF of crude oil by teleost fish has been reported to cause alterations that compromise their survival in the particular environment. The hydrocarbons derived from the oil cause structural damages to the respiratory lamellae of fish gills. Moreover, components of the oil are capable of causing genetic damages, alterations in reproductive and nutritional behaviors, and cellular aberrations (Billiard et al. 2006). Biochemical, physiological and histological biomarkers have been used to determine the effects of petroleum-derived hydrocarbons in aquatic biota (Simonato et al. 2008; Silva et al. 2009).

Xenobiotic-biotransformation enzymes belonging to the cytochrome P450 (CYP) family play central roles in the oxidative metabolism of a wide range of exogenous and endogenous compounds. Alteration of the expression of hepatic CYPs markedly affects the potential risks and benefits of xenobiotics and is important from a toxicological point of view because of the roles of this class of enzymes in the detoxification and activation of foreign compounds (Williams et al. 1998). The most common biomarker used to measure CYP1A is the ethoxyresorufin-O-deethylase (EROD) activity. The phase II enzyme glutathione S-transferase (GST) plays a role in the detoxification of oxidative stress products as well as in the conjugation of glutathione to xenobiotic metabolites to facilitate their excretion.

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According to Hallare et al. (2005), studies with zebrafish eggs have gained importance in the last few years because these are used both to evaluate the effects of chemical compounds and to determine the maximum allowable concentrations of solvents that can be used in experimental systems. *Danio rerio* possesses many advantages, such as a short generation time, high fecundity, rapid development, external fertilization, and translucent embryos, facilitating its use as a test model (Westerfield 2000).

Therefore, the aim of the present study was to evaluate the biotransformation and embryotoxicity of different concentrations of the WSF of crude oil in relation to zebrafish.

Materials and Methods

The oil used in the experiments (API27) was obtained from Campos Bay (Rio de Janeiro, Brazil). The aromatic hydrocarbons comprised about 27% of the oil, 55% were saturated hydrocarbons, 16% resin hydrocarbons and 1.6% asphaltenes.

The WSF of the crude oil was prepared according to Anderson et al. (1974) by pouring 1 part of oil above 9 parts of reconstituted water (0.1335 g L^{-1} $MgSO_4$; 0.0004 g L^{-1} KCl; 0.0065 g L^{-1} $CaCl_2$; 0.0105 g L^{-1} $NaHCO_3$; pH 7.2–7.3) in a Pyrex bottle and slowly stirring the water at 20 rpm for 20 h at 20.0 ± 2.0°C. To avoid evaporation of the volatile hydrocarbons, the bottle was capped with a plastic foil, and a black plastic was used for wrapping the bottle to avoid the interference of light. After mixing, the oil and the water-soluble portion, designated the 100% soluble fraction, were separated. Different concentrations (15%, 33%, and 50%) were prepared in individual reservoir flasks from the 100% stock solution, and were used in the experiments. The concentrations were based in Akaishi et al. (2004).

A gas chromatographic analysis of the total petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAHs) and aliphatic hydrocarbons of the 100% fraction was carried out in the Technology Institute at Paraná Federal University based on the method by Bícego et al. (2006). Solid phase extraction (SPE) was based on Carichia et al. (1999). The hydrocarbon extracts were fractionated into aliphatics and aromatics by silica gel–alumina column chromatography. Aliphatic hydrocarbons were determined on a Hewlett Packard 5890 II high-resolution gas chromatograph equipped with flame ionization detector (GC-FID). PAHs were analysed by an Agilent 6890 gas chromatograph coupled to a 5973 N mass spectrometer (GC–MS) in the selected ion monitoring (SIM) mode.

Sexually mature zebrafish (*Danio rerio*) specimens were maintained in 15-L aquaria (25.0 ± 0.5°C and pH of 7.0 ± 0.2) under a 12 h light/12 h dark photoperiod. They were fed twice daily with commercially available artificial diet (Tetramin flakes) and *Artemia* sp. A grid was installed within the aquarium to prevent the fish from eating the eggs. According to the report by Westerfield (2000), six fish were housed per aquarium in the ratio of 1 male to 2 females. All the procedures in this study were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (Canadian Council on Animal Care), and the animal protocol was approved by the ethical committee for animal experimentation of the Paraná Federal University.

A solution of 2% ethanol, obtained by mixing 99.9% ethanol and distilled water, was used as a positive control.

The eggs were collected in the morning and rinsed several times with reconstituted water. After a few regular cleavages, fertilized eggs were selected for exposure. They were subsequently transferred to Petri plates containing either different concentrations of the WSF of crude oil, 2.0% ethanol, or reconstituted water to observe embryo development. Batches of 10 zebrafish eggs were introduced into each plate; totally, 9 chambers were used. The experiment was repeated twice. The growth media were replaced every 24 h, and the incubation temperature was maintained at 28.5°C. The development of the embryos was monitored for 96 h after following fertilization. During the observation period, dead embryos were removed to avoid contamination of the surviving ones. Data regarding embryo mortality, tail and eye defects, pigmentation, movements and heartbeat for each group were recorded using a microscope. The heartbeat was considered reduced when it was less than 160 bpm. The group unexposed to WSF of crude oil recorded approximately 180 bpm.

For the analysis of GST and EROD activities, 2-week-old juvenile zebrafish were exposed in 15-L aquaria to the three different concentrations of crude oil WSF in reconstituted water (n = 34). The fish were not fed during the exposure period. After 96 h, the fish were killed with 2% benzocaine, the heads and tails removed, and the bodies (pools of 2 animals) stored at −70°C.

The samples were homogenized with 2 mL of phosphate buffer (pH 6.5) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 30 min at 10,000×*g* at 4°C. The supernatant was aliquoted into different containers to study the enzymes. The EROD activity was measured on the basis of the method of Burke and Mayer (1974). The measurement was carried out with a Shimadzu spectrofluorimeter using 530 and 590 nm as the excitation and extinction wavelengths, respectively. Enzyme activity was expressed as pmol resorufin. min^{-1} mg^{-1}protein. The GST activity was measured at 340 nm by the method described by Keen et al. (1976). The activity was expressed in nmol min^{-1} mg^{-1}protein. Protein concentration was

determined using Bradford's method (1976) with bovine serum albumin as the standard.

The enzymatic data were expressed as mean values ±standard error (n = 17). The embryotoxicity data were expressed in percentage, based on the method of Hallare et al. (2006). They were tested for normality using the Shapiro–Wilk test y; the one-way analysis of variance (ANOVA) was used to test the differences between the groups, followed by Bonferroni's test. T-test was used to compare the ethanol group (positive control) with the negative control group. Differences were considered significant when $p < 0.05$.

Results and Discussion

From chemical analysis, the 100% concentration of crude oil WSF was considered to consist of 442.5 mg/L of TPH, 119.5 mg/L of PAHs (27%), 323 mg/L of aliphatic hydrocarbons (63%). The concentrations of WSF of crude oil used in this study sought to reproduce the real circumstances of an accident involving oil. In this type of accident, different processes occur that change the oil characteristics, and its products are more toxic in certain instances. During dispersion, the oil spill is broken into small patches or globules, increasing the surface contact with water. The dissolution and dispersion of the WSF compounds are important chemical processes because they remain in the water even after the oil spot is removed.

In the present study, the embryotoxicity of three different concentrations (15%, 33%, and 50%) of WSF from crude oil was determined, with ethanol as the 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 a high yield of synchronously fertilized eggs, transparency of embryos and rapid embryonic development. Loucks and Carvan (2004) showed alterations in neurocranial and craniofacial skeletal development and growth retardation when zebrafish embryos were exposed to ethanol, and they related its effects to those in humans.

All of the test groups in the present study were compared to a negative control group which was not exposed to the WSF. The developmental stages of the no-exposure group were normal, similar to those described in literature. After approximately 4 h of development, a prominent cap of regular small cells was formed above the yolk and, at 24 h, the basic vertebrate body organization was observed, in association with the commencement of heartbeat and spontaneous movements. The completion of rapid morphogenesis of primary organ systems and cartilage development in both the head and the pectoral fins occurred at 48 h. Spontaneous movements started at 24 h of development, and the black pigmentation of the embryos was apparent macroscopically. The hatching success during the experiment was normal, and the 96-h survival rate was sufficiently high. The negative control group showed a normal embryonic development, with concurrent formation of all the structures (Fig. 1). The negative control group had a heartbeat rate of 180–200 bpm.

The ethanol-treated embryos also showed toxic effects, ethanol being a good positive control. The hatching process was delayed compared to the negative controls; in some cases, a total absence of hatching was observed.

Embryos exposed to crude oil WSF were adversely affected at the three concentrations studied (15%, 33%, and 50%), a significant number of embryos died during exposure. The heartbeat of the specimens was reduced at all the three concentrations, but there was no significant difference between the 33% and 50% exposure groups (Table 1). Exposure to weathered crude oil or individual tricyclic PAHs (fluorene, dibenzothiophene, and phenanthrene) causes specific defects in cardiac rate, rhythm, and contractility soon after the heart becomes functional (Incardona et al. 2005). Because cardiac function and morphogenesis are inextricably linked (Glickman and Yelon 2002), these effects of oil exposure result in multiple secondary impacts, such as failure to finish looping in cardiac morphogenesis. Moreover, mechanistic studies

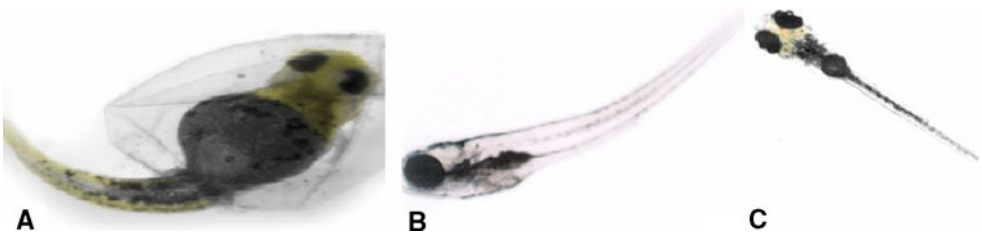

Fig. 1 Embryonic development of *Danio rerio* at 72 h. **a** 72-h embryo with abnormal body structure and tail defect after exposure to water-soluble fraction of crude oil (33%); **b** and **c** negative control embryo with well-developed structure (at 72 h), in lateral and dorsal views, respectively

Table 1 Alterations in zebrafish embryos after exposure to different concentrations of the water-soluble fraction of crude oil and control groups

Development defects	Negative control	WSF 15%	WSF 33%	WSF 50%	Ethanol 2%
Tail defects	0[a]	11–12[a]	15–17[a]	20–23[b]	23–25*
Reduced heartbeat	0[a]	87–90[b]	90–93[c]	90–93[c]	60–64*
Weak pigmentation	0[a]	62–64[b]	83–85[c]	90–92[d]	34–36*
Eye defect	0	0	0	0	0
Mortality	10–12[a]	65–67[b]	75–80[c]	88–93[d]	89–92*

Data are expressed in percentages of two experiments

Different letters indicate significant differences among the treatments (ANOVA-Bonferroni, $p < 0.05$)

* = $p < 0.05$ compared to negative control group (t-test)

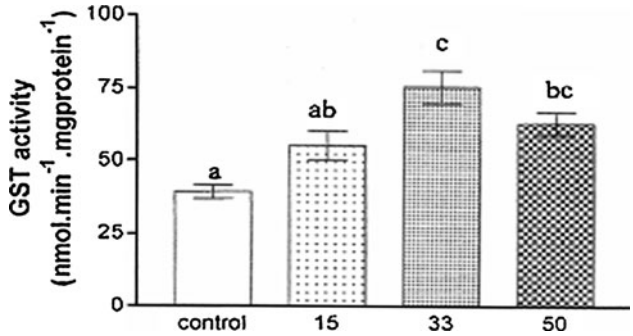

Fig. 2 Glutathione S-transferase activities in *Danio rerio* exposed to different concentrations of the water-soluble fraction of crude oil (15%, 33%, and 50%) and in the negative control group. Different letters indicate significant differences among the treatments ($p < 0.05$)

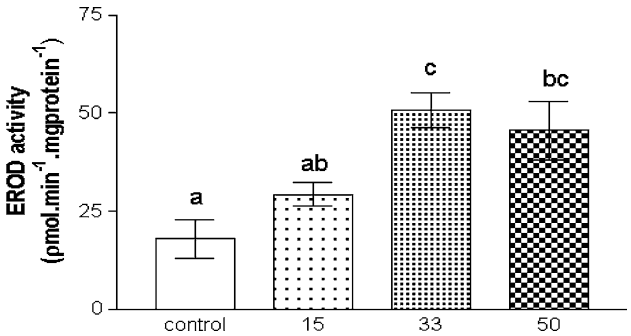

Fig. 3 Ethoxyresorufin-O-deethylase activity in *Danio rerio* exposed to different concentrations of the water-soluble fraction of crude oil (15%, 33%, and 50%) and in the negative control group. Different letters indicate significant differences among the treatments ($p < 0.05$)

show that PAH constituents in crude oil are cardiotoxic to fish embryos whether present as pure compounds or in oiled rock-column effluents (Incardona et al. 2005).

Pigmentation was inversely related to WSF concentration. At the 15% concentration, approximately 64% of the embryos showed weak pigmentation; whereas at 33% and 50%, the percentages of embryos showing weak pigmentation were 85% and 92%, respectively. Tail deformities were statistically significant at the high concentration, occurring at a lower percentage as compared to the control embryos. No defects were observed in the eyes. During the observation period, the embryos exposed to the WSF of crude oil were not responsive to stimulations and, similar to the ethanol-exposed embryos, showed no spontaneous movements.

In juvenile fish exposed to 33% and 50% concentrations of the WSF of crude oil, the activities of EROD and GST increased significantly compared with the negative control group. There were no significant differences between the 33% and 50% group but there were differences between the 15% and 33% exposure groups (Figs. 2 and 3). Several studies have shown that the degree of CYP1A induction in fish can be closely linked to the degree of contamination by chemicals, such as polychlorinated biphenyls and PAHs, found in the animals themselves or in other biota or sediments at the same site (Goksøyr et al. 1994).

GST catalyzes the conjugation of eletrophilic xenobiotics to GSH and plays an important role in protecting tissues from oxidative stress. Jifa et al. (2005) affirm that GST responds differently after exposure to different compounds. GST has been shown in fish to be involved with the elimination of the WSF from oil. Zhang (2004) observed that in the goldfish, *Carassius auratus,* exposure to the WSF of diesel oil resulted in an increase in GST activity at higher concentrations of PAHs compared to that at lower concentrations. Moreover, GST is also induced in organs other than the liver, with extrahepatic GST activity having been shown in a number of species; the other organs involved were the intestine and gills (George 1994).

As shown in this study, the water-soluble fraction of crude oil altered the normal embryonic development of fish. Consequently, fish species that reproduce and have their embryos, fry and juveniles in oil spill–affected areas are likely to be diminished in population. It is also important to remember that in addition to accidents, there are other modes of oil contamination, such as wastewaters from refineries and oil-contaminated industrial effluents. Aquatic organisms, mainly those inhabiting the coastal

regions, invariably suffer hydrocarbon contamination, stressing the importance of both monitoring of their populations and enforcement of pollution-control mechanisms to ensure adequate survival of these species.

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