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Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: Studies with *Prestige* oil and medaka (*Oryzias latipes*)

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are assumed to be the primary determinant of oil petroleum toxicity. Since the PAH content in *Prestige* oil was relatively high, we investigated the effects of different oil fractions (crude or weathered oil −0.05 to 50 g/L, and shaken or sonicated water accommodated fractions, WAFs, 25–100%, v/v) on the embryo–larval development of medaka (*Oryzias latipes*). Concentrations of $\sum$16PAHs analyzed in the incubation medium were highest in the shaken WAF followed by the crude oil, the sonicated WAF and the weathered oil. Both oils ($\geq$0.25 g/L) induced developmental abnormalities whereas no significant effects were seen in the WAF exposures. *In vivo* morphometric analysis of the surface of the gallbladder during advanced embryo organogenesis (192 h post-fertilization, hpf) revealed significant dilation in both WAF exposures ($>3 \times 10^4$ μm^2 at $\geq$25%, v/v, compared to $<1.7 \times 10^4$ μm^2 at 0%, v/v) followed by the crude oil ($>2.2 \times 10^4$ μm^2 at $\geq$0.05 g/L). Fluorescent aromatic compounds were observed in the gallbladder and the yolk sac of 168-hpf embryos exposed to all oil fractions. Results suggest the presence of components in both oils capable of penetrating the chorion and inducing a toxicity not observed in the WAFs. Hence, the hazard and risk assessment of *Prestige* oil should not be based solely on the presence of PAHs since proximity or direct contact may induce toxicity not associated exclusively to these compounds. This research offers a new hypothesis for explaining the reported biological observations, which could be correlated to direct oil exposure rather than the traditional mechanism of waterborne PAH exposure. Further research is needed to identify those oil components responsible for toxicity.

1. Introduction

Oil spills caused by maritime transport of petroleum products are an important source of marine and particularly coastal pollution along main transport routes. Among the components in an oil spill, those causing with most concern regarding environmental risk are the polycyclic aromatic hydrocarbons (PAHs) as they exhibit high toxicity in the aquatic environment (Kocan et al., 1996; Incardona et al., 2004; Lee and Anderson, 2005). Greater acute toxicity is generally associated with the lower molecular weight PAHs, whereas some high molecular weight PAHs form metabolites that can function as carcinogens (Overton et al., 1994; González et al., 2006).

The sinking of the tanker *Prestige* in 2002 was another in a series of major oil tanker disasters which seem to occur every year or so. The wreck resulted in the release of 64,000 tonnes of Russian heavy fuel-oil into the northwestern Iberian shelf causing immediate impact on large areas of the coast affecting all activities linked to mariculture and fishing, as well as affecting the ecological and tourist value of various stretches of coast (IEO, 2003; IOPC Funds, 2003; Albaigés et al., 2006). As a consequence, the Spanish Government approved a Strategic Action and mid-term Scientific Response Plan for the hazard evaluation and remediation of the affected area. Analytical studies carried out by the Spanish National Research Council (CSIC, 2003) revealed a low volatility of this fuel-oil due to its relatively elevated content of medium to high molecular weight hydrocarbons, including PAHs. Numerous investigations have covered diverse aspects including oil fate (Medina-Bellver et al., 2005; González et al., 2006) and a toxicological characterization using coastal organisms such as mussels (Cajaraville et al., 2006), sea urchins (Fernández et al., 2006), seabirds (Oropesa et al., 2007), and fish (Martínez-Gómez et al., 2006; Morales-Caselles et al., 2007). Specifically for this last group, Sánchez et al. (2006) evaluated changes in the abundance on native key species such as the European hake (*Merluccius merluccius*) and the fourspotted megrim (*Lepidorhombus boscii*) in affected areas by the spill. However, the impacts of oil spills on aquatic populations are difficult to measure since individuals suffering from sublethal effects may not be identi-

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fied and counted among the mortalities, even though this situation compromises the viability of the individual (Heintz et al., 2000). Martínez-Gómez et al. (2006) have reported sublethal effects such as elevated phase I and phase II enzymatic activities in two demersal species, fourspotted megrim and dragonet (*Callionymus lyra*), inhabiting the affected areas. Nevertheless, the presence of background non-point pollution in the area prevented from attributing any response exclusively to the *Prestige* oil spill (POS).

Besides its relatively high content of PAHs, the *Prestige* fuel-oil showed a high density which increased the relative role of potential effects associated with direct contact instead the waterborne exposure to the water accommodated fraction (WAF). In addition, as this type of oil ends up accumulating in sediments quickly, the impacts to fish are primarily expected on demersal species and particularly on eggs and larvae (Overton et al., 1994; Brannon et al., 2006), and sublethal impacts that are normally difficult to measure would be most profound in populations exposed during early developmental stages (Rosenthal and Alderdice, 1976).

Using embryo larval stages (ELS) of the Japanese medaka fish (*Oryzias latipes*) and *Prestige* fuel-oil, we compared the toxicity of two types of WAFs obtained with that observed by a direct exposure to crude or weathered oils. A controlled exposure of fish eggs to crude or weathered oils may be particularly interesting as the consequences of physical disturbances are minimized when compared to free swimming organisms. Exposures were performed during the whole embryo development and daily observations were made on embryos and surviving larvae. We selected specific toxicity endpoints (i.e. embryo and larval malformations, hatching success, swimming activity and larval length) and exposure endpoints (i.e. *in vivo* determination of the embryonic gallbladder outlined surface and presence of fluorescent aromatic compounds, FACs), and evaluated potential correlations. The present findings will aid in understanding the importance of evaluating the different fractions of a fuel-oil as a significant modifying factor of toxicity to fish ELS and the need for consideration of this factor in the environmental risk assessment of areas impacted with an oil spill.

2. Materials and methods

2.1. Sample collection

We obtained fresh crude oil (Co) samples from the Technical Office for Marine Spillages at the University of Vigo, Galicia (OTVM, 2003). These samples and the Co originally carried by the *Prestige* tanker shared identical physical and chemical properties. Weathered oil (Wo) samples were collected in an affected shoreline of the Bay of Biscay weeks after the spill. Any residual water in the Wo samples was removed by allowing the oil and water to separate during refrigeration. All samples were stored in our laboratory in hermetic containers refrigerated at 4 °C until use in toxicity testing and in the preparation of the WAFs.

2.2. Water accommodated fractions

We prepared WAFs from Co samples in sealed 500 ml carboys using 5 g of oil per 100 ml of embryo rearing medium (RM) (Rugh, 1962). From previous studies in our laboratory (Navas et al., 2006), higher oil-to-dissolving medium ratios did not increase the total petroleum hydrocarbons content. Two types of WAFs, a shaken WAF (shWAF) and a sonicated WAF (soWAF) were prepared. To obtain the shWAF, carboys were shaken in darkness for 48 h in an orbital shaker (40 rpm). For the soWAF, carboys were initially sonicated for 15 min before the 48-h shaking. All WAFs were then allowed to sit for 2 h, passed through a 0.44 μm polyamide filter, the air space of the carboy filled with nitrogen, sealed with Teflon lids and stored at 4 °C.

2.3. PAH characterization

The 16PAHs ($\sum$16PAHs) recommended in fuel-oil analysis by the US EPA (Keith and Telliard, 1979) were evaluated in the shWAF and soWAF fractions at 100% (v/v) after incubation for 1 day, and in the Co and Wo fractions at 5.00 g/L after incubation for 7 days in sealed vials under the same conditions as in the embryo exposures (see below). Three replicates were included for each of the fractions and incubation periods. PAHs were determined according to the EPA 610 method for organic chemical analysis of municipal and industrial wastewater (US EPA, 2006). Briefly, 125 ml of samples were extracted by solid phase extraction (Strata-X 33 μm polymeric reversed phase 60 mg/3 ml, 8B-S100-UBJ, Phenomenex, Torrance, CA) previously conditioned with 5 ml of dichloromethane, 5 ml of methanol, and 5 ml of MilliQ water (Millipore, Billerica, MA). After sample absorption the cartridge was washed with 5 ml of MilliQ water and dried under vacuum for 60 min. PAHs were then desorbed with 10 ml of dichloromethane. The organic extraction was evaporated at 40 °C (EZ-2 Genevac, Ipswich, United Kingdom) and redissolved in 1 ml of acetonitrile. PAHs were analyzed by high performance liquid chromatography in a 2690-Waters Alliance liquid chromatograph (Waters, Milford, MA) with a 776-Waters fluorescence detector and a diode array detector, injecting 100 μl of sample in a Envirosep PP 4.6 mm × 125 mm column (Phenomenex) at 40 °C with an acetonitrile/MilliQ water gradient at 1.2 ml/min for 60 min. Quantification was performed with an external standard using SS EPA 610 PAH Mix (4S8743 Supelco, Sigma Chemical Co., St. Louis, MO) to built the corresponding calibration curve.

2.4. Experimental design

Golden medaka embryos were obtained from our own broodstock held since 2004. Culture conditions were as described elsewhere (González-Doncel et al., 2005). Naturally fertilized eggs, collected after the start of the daylight cycle were immersed in RM and allowed to develop at 25 °C to the late morula stage (i.e. stage 9 or 5 h post-fertilization, hpf; Iwamatsu, 1994; González-Doncel et al., 2005) for subsequent exposures.

Four different toxicity tests were performed. In the first two tests, eggs were exposed to concentrations of shWAF or soWAF (values of 25, 50 and 100%, v/v). In the next two experiments, embryos were exposed to a range of five experimental solutions of Co or Wo (0.05, 0.25, 0.50, 5.00 and 50.00 g/L). WAF-experiments were run as static 24-h renewal while Co- and Wo-experiments were run as static non-renewal exposures. In all experiments, eggs (5 hpf/25 °C) were exposed for the duration of their complete embryonic development (240 hpf/25 °C). Each bioassay was conducted as a completely randomized design in which embryos were distributed ($n = 8$) in each of five replicates by stratified random assortment into 20-ml borosilicate vials (Wheaton Scientific, Millville, NJ) with 2 ml of test solution and sealed with Teflon-lined lids. Controls were exposed to an equal volume of RM alone. For each experiment, vials were coded for blind study except for an additional RM replicate (known control). Conditions for incubation of exposures followed those previously described (González-Doncel et al., 2005). After exposures, surviving individuals were rinsed with RM and transferred to clean vials where they were allowed to complete development and hatch. Individual hatchlings were collected daily, placed in new vials where the RM was renewed every 24 h, and developmental evaluation was continued through the following 72 h without any exogenous food supply. Based on our experience, the yolk sac contains sufficient nutrients for 3 days after hatching.