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Evaluation of artificially-weathered standard fuel oil toxicity by marine invertebrate embryogenesis bioassays

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HIGHLIGHTS

- We describe an increase in marine ecotoxicity of weathered oil, in contrast with the classic paradigm.
- We prove by using analytical chemistry that the increased toxicity is not related with the total aromatic content.
- Formation of oxidized derivatives of parental aromatics might account for the increase in toxicity throughout weathering.

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ABSTRACT

Weathering of petroleum spilled in the marine environment may not only change its physical and chemical properties but also its effects on the marine ecosystem. The objective of this study was to evaluate the toxicity of the water-accommodated fraction (WAF) obtained from a standard fuel oil following an environmentally realistic simulated weathering process for a period of 80 d. Experimental flasks with 40 g L^{-1} of fuel oil were incubated at 18 °C with a 14 h light:10 h dark photoperiod and a photosynthetically active radiation (PAR) intensity of 70 μE m^{-2} s^{-1}. Samples were taken at four weathering periods: 24 h, 7, 21 and 80 d. WAF toxicity was tested using the sea urchin (*Paracentrotus lividus*) and mussel (*Mytilus galloprovincialis*) embryo–larval bioassays and the aromatic hydrocarbons levels (AH) in the WAF were measured by gas chromatography/mass spectrometry. In contrast with the classic assumption of toxicity decrease with oil weathering, the present study shows a progressive increase in WAF toxicity with weathering, being the EC_{50} after 80 d eightfold lower than the EC_{50} at day 1, whereas AH concentration slightly decreased. In the long term, inoculation of WAF with bacteria from a hydrocarbon chronically-polluted harbor slightly reduced toxicity. The differences in toxicity between fresh and weathered fuels could not be explained on the basis of the total AH content and the formation of oxidized derivatives is suggested to explain this toxicity increase.

1. Introduction

Every year more than 14,000 ships transporting hazardous substances follow one of the main maritime transport routes that link Europe with America and Asia, located just a few tens of miles off Galicia (NW Iberian Peninsula). Since the 1950s, some of the most important accidents of oil tankers, such as *Andros Fortune*, *Polycommander*, *Urquiola*, *Andros Patria*, *Aegean Sea* and *Prestige*, have occurred in this area (CEDRE, 2008). Concurrently, the Galician coast is a highly productive area where shellfishing and aquaculture bear both social and economic importance (Pérez-Camacho et al., 1995; Labarta and Corbacho, 2002). Therefore, the study of the impact of oil spills on representative marine species deserves particular interest.

Petroleum and their derivatives affect organisms by physical action (e.g. light reduction, asphyxia), by modification of habitats (e.g. decrease of dissolved oxygen, decrease in food availability) and by toxic effects of soluble or emulsionable components. The most relevant effects of oil are attributed to the aromatic fraction, which has been considered potentially mutagenic, carcinogenic and teratogenic (Albers, 2003; Penela-Arenaz et al., 2009).

When a petroleum product, either crude or refined, is spilled at sea, weathering processes including spreading, evaporation, dissolution, dispersion, emulsification, photochemical oxidation, microbiological degradation, absorption in suspended particles and sedimentation, change the physical and chemical properties of the product and also its effects on the marine ecosystem (Kennish,

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1992). Toxicity changes depend on the physicochemical characteristics of the petroleum product and the predominant weathering process. Several authors have studied the change on the toxicity of crude oils in order to validate the classic assumption of toxicity decrease with oil weathering (Carls et al., 1999; Neff et al., 2000; Perkins et al., 2003; Barron et al., 2005). However, since those studies have used different weathering treatments, it has not been found a clear pattern of increase or decrease of oil toxicity. Moreover, most of them use weathering treatments such as heating or distillation which are environmentally not realistic (Neff et al., 2000; Perkins et al., 2003; Barron et al., 2005).

Early life stages are more sensitive to toxicants than adults, and represent a critical period in the life cycle of an organism. Moreover, unlike larger, actively swimming organisms, larvae are part of plankton, and they lack the ability to escape from oil-polluted waters. Sea urchins (Falk-Petersen, 1979; Fernández et al., 2006; Saco-Álvarez et al., 2008) and molluscs (Pelletier et al., 1997; Mariño-Balsa et al., 2003; Saco-Álvarez et al., 2008) are among the most frequently used organisms in embryo–larval bioassays to determine the toxicity of oil products and, since those are water-column organisms, a liquid-phase must be obtained. According to the recommendations of the European Centre of Ecotoxicology and Toxicology of Chemical Substances (ECETOX), the exposure medium for toxicity tests of low solubility compounds, as fuel oil, should be prepared as a water-accommodated fraction (WAF) (Rufli et al., 1998). WAF is defined as a medium containing only the fraction of petroleum that remains in the aqueous phase once any source of mixing energy has been removed and after a period sufficient for phase separation. WAF is considered to be equilibrated with petroleum products, mainly hydrocarbons that can be present in true solution or like a stable emulsion (Rufli et al., 1998).

Due to the lack of information in literature on the toxicity of products derived from crude oil refining to marine organisms, the aim of this work was to study the evolution of toxicity of a standard fuel oil subjected to an artificial weathering process aimed at reproducing some of the physicochemical processes that take place in the marine environment. These processes included aeration, light exposure and bacterial inoculation, for a period of 80 d. Toxicity was tested by obtaining the WAF from the weathered fuel oil and using two reliable and sensitive bioassays, the bivalve and sea urchin embryogenesis bioassays.

2. Materials and methods

2.1. Fuel oil characterization

The product tested was "Marine fuel oil", a standard intermediate fuel oil (ISO 8217, IFO 380, RMG 35) which is a blend of gas oil and heavy fuel oil, characterized by a density of 0.98 kg L^{-1} at 15 °C and a viscosity at 50 °C of 380 cSt (ISO, 2005). Fuel oil was supplied for scientific research by the Spanish Government through the *Oficina de Vertidos Marinos Accidentales* (Universidade de Vigo, Spain).

2.2. Fuel oil weathering process and WAF preparation

Weathering experiments were performed by adding 40 g of fuel oil to 1 L of 0.22 μm-filtered sterile seawater (FSW) in 2 L glass flasks. An additional set of flasks was prepared and inoculated with bacteria from a site in Vigo Harbour chronically contaminated by hydrocarbons, in order to detect possible effects of the bacterial degradation of hydrocarbons on the WAF toxicity. The inoculate was prepared by filtering 1 L of seawater collected in Bouzas harbour through a 0.22 μm sterile filter, and transferring the bacteria by agitating the filter into 250 mL sterile seawater. Both sets of flasks covered with aluminium foil were magnetically stirred with a Teflon-coated stirring bar of triangular section at an arbitrary constant speed (Tsvetnenko and Evans, 2002). Experimental flasks were incubated in a culture chamber at 18 °C with a 14 h light:10 h dark photoperiod and a photosynthetically active radiation (PAR) intensity of 70 μE m^{-2} s^{-1}. Cool daylight lamps (Osram 15W/765), with an emission spectrum range of 380–780 nm were used in order to simulate natural irradiation. Four weathering periods were tested (24 h, 7, 21 and 80 d), using one flask of each treatment per weathering time.

After the weathering period, WAF was obtained by filtration through a glass fibre filter (Millipore APFF04700). WAF dilutions for toxicological testing were varied on the basis of the expected toxicity for each sampling time. Dilutions were prepared with non-sterile 0.22 μm-filtered seawater and dosed (20 mL) in 25 mL glass vials with Teflon-lined caps. Five replicates per treatment and five FSW controls were assayed for each experiment. Vials were incubated in a culture chamber at the temperature and photoperiod conditions explained above. WAF samples for chemical analysis were collected in brown glass bottles and acidified (pH < 2) to avoid biodegradation. WAF samples of 5 mL were also collected for bacteria count in sterile vials, fixed by adding 40% buffered formalin in a proportion 50 mL L^{-1} and stored at 4 °C in darkness.

2.3. Biological material

Mature sea urchins (*P. lividus*) were collected in pristine sites from local populations in the Ría de Vigo (Galicia, NW Iberian Peninsula) and mature mussels (*Mytilus galloprovincialis*) were purchased at the local market in Vigo. Animals were transported to the laboratory in a portable icebox and maintained in aquaria with running natural seawater until the experiments were carried out.

2.4. Mussel bioassay

Mussels were induced to spawn by thermal stimulation in separated beakers with FSW following Bellas et al. (2005). Eggs from a single female were transferred to a 100 mL measuring cylinder and their quality was checked under microscope. Sperm solution was stored at 4 °C until use. Sperm mobility was checked under microscope and a few μL were added to the egg suspension and carefully stirred to allow fertilization. Fertilized eggs (ca. 30 eggs mL^{-1}) were transferred to vials containing the experimental solutions and incubated for 48 h. After the incubation period, larvae were preserved by adding a few drops of 40% buffered formalin and the percentage of D-veliger larvae (n = 100) was recorded.

2.5. Sea urchin bioassay

Methods were based on Saco-Álvarez et al. (2010). Gametes were obtained by dissecting mature sea urchin specimens. Gamete quality (round eggs and motile sperm) was checked under the microscope. Eggs were transferred into a 50 mL measuring cylinder containing 0.2 μm-filtered seawater. A few μL of undiluted sperm were added and the contents of the cylinder were gently stirred with a plunger for 2 min approximately to allow fertilization. Four 20 μL samples were taken by a pipette and observed under the microscope in order to record the number of eggs and fertilization success (indicated by the presence of a fertilization membrane). Within 30 min after fertilization, fertilized eggs (c.a. 25 eggs mL^{-1}) were transferred to vials containing the experimental solutions, incubated for 48 h and then fixed by adding a few drops of 40% buffered formalin. The recorded response was growth, defined as the maximum dimension in the first 35 individuals per vial (including embryos), subtracting the average of maximum dimension fertilized eggs. Length of individuals was recorded in