Marine Pollution Bulletin 57 (2008) 493–502

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Ecotoxicological evaluation of polycyclic aromatic hydrocarbons using marine invertebrate embryo–larval bioassays

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ARTICLE INFO

Keywords:
Polycyclic aromatic hydrocarbons
Marine invertebrates
Embryo–larval bioassays
Phototoxicity
Mixture toxicity

ABSTRACT

The toxicity of polycyclic aromatic hydrocarbons (PAHs) was determined using mussel, sea-urchin and ascidian embryo–larval bioassays. Fluorescent light exposure enhanced phenanthrene, fluoranthene, pyrene and hydroxypyrene toxicity in comparison with dark conditions, but not naphthalene and fluorene toxicity. The toxicity of PAHs was inversely related to their K_{OW} values following QSAR models derived for baseline toxicity of general narcotics, whereas the obtained regression using toxicity data from photoactivated PAHs significantly departed from the general narcosis model. Also, the mixture toxicity of five PAHs to the larval growth of the sea-urchin was compared with predictions derived from the concentration addition concept, indicating less than additive effects. Finally, we compared our toxicity data with worst-case environmental concentrations in order to provide a preliminary estimate of the risk to the marine environment. Naphthalene, fluorene and pyrene are not considered to pose a risk to sea-urchin, mussel or ascidian larvae, whilst phenanthrene and fluoranthene may pose a risk for mussel and sea-urchin. Moreover, a higher risk for those species is expected when we consider the photoactivation of the PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of widespread organic compounds of high environmental concern. Even though PAHs occur naturally, the highest concentrations are mainly due to human activities that cause a continuous increase in PAH levels of estuarine and marine waters (Kennish, 1992; Walker et al., 2001). Direct discharges into the marine environment from point sources such as wastewater treatment plants range from <1 μg/L to over 625 μg/L, whilst concentrations of PAHs in industrial effluents range from undetectable to 4.4 mg/L (Latimer and Zheng, 2003). Major sources of PAHs to the marine environment are combustion products and petroleum principally from atmospheric deposition (5×10^4 tonnes/year) and oil spillage (1.7×10^5 tonnes/year) (Kennish, 1992; Meador, 2003). For instance, in November 2002 the oil tanker *Prestige* sank 130 miles off the Galician coast (NW Spain), spilling more than 60,000 tonnes of number 2 fuel oil into the open sea (CEDRE, 2007). This is not an isolated disaster, since Galicia has received nine oil spills during the last 50 years (CEDRE, 2007), being one of the regions with the highest number of oil spills in the world. Galician Rías are highly productive estuaries well known because of its coastal fishing and shellfish production. Among the shellfish production of the Galician Rías stands out the culture of the mussel (*Mytilus galloprovincialis*) as the main cultivated species, with an annual production of more than 2.5×10^5 tonnes (1.3×10^8 euros) (Pérez-Camacho et al., 1995; Labarta and Corbacho, 2002). Therefore, it is necessary to study the impact of those pollutants to marine organisms of ecological and commercial relevance. Furthermore, growing evidence suggests that solar radiation may enhance the toxicity of certain PAHs. Some PAHs can absorb the radiation and become photoactivated, the photoactivated molecule may then transfer the radiation energy to molecular oxygen thereby forming reactive superoxide anions capable of oxidative damage in the organisms (Landrum et al., 1986; Arfsten et al., 1996).

The present study focussed on the toxicity assessment of low and intermediate molecular weight PAHs (2–4 rings), since they constitute the most toxic components of oil for the marine biota, using a battery of sensitive embryo–larval bioassays with marine organisms of commercial and ecological relevance. The specific goals of the present study were to investigate the toxicity and potential phototoxicity (under fluorescent light and in dark conditions) of selected PAHs individually and in mixtures, based on current models of narcotic toxicity. Bioassays with marine organisms intended to be used in the assessment of marine pollution must meet some fundamental requirements: (i) ecological relevance, using ecologically relevant or commercial organisms; (ii)

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0025-326X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2008.02.039

feasibility, the bioassays must be easy to standardise, based on precisely defined protocols, and using simple, rapid and cost-effective bioassays; and (iii) sensitivity, using for instance, early life stages of development, which are less tolerant to toxicants than adults (e.g. Connor, 1972; Marin et al., 1991; Ringwood, 1991; His et al., 1999), and sublethal biological responses. We conducted embryo-larval bioassays with the bivalve *M. galloprovincialis*, the echinoid *Paracentrotus lividus* and the ascidian *Ciona intestinalis*. Those species were chosen due to their commercial importance, their abundance and their importance in the functioning of the marine ecosystem (Dybern, 1965; Gosling, 1992; Boudouresque and Verlaque, 2001).

2. Materials and methods

2.1. Biological material

Mature *C. intestinalis* and *P. lividus* were collected in pristine sites from local populations in the Ría de Vigo (Galicia, NW Spain). Mature *M. 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 for a least one week. Handling conditions of the adult stock were 17.43 ± 0.54 °C temperature, 35.01 ± 1.52 ppt salinity, 7.24 ± 1.14 mg l^{-1} O_2 and 7.88 ± 0.09 pH (mean ± std).

2.2. Experimental solutions

Stock solutions were made by dissolving analytical grade naphthalene, phenanthrene, fluoranthene, fluorene, pyrene and hydroxypyrene (Sigma–Aldrich, Steinheim) in acetone, due to the low solubility of PAHs in seawater (Kennish, 1992). The experimental concentrations were obtained by diluting the stock solutions in artificial seawater (ASW) prepared as in Zaroogian et al. (1969). During this dilution, equal amounts of acetone (less than 200 μl L^{-1}), found not to be toxic in preliminary tests, were added to each experimental beaker with PAHs solutions. All glassware was acid-washed (HNO_3 10% vol.) and rinsed with acetone and distilled water before the experiments.

Experimental concentrations were chosen on the basis of range-finding trials and on data from the literature. Tested concentrations for each compound were below their water-saturation levels (Nagpal, 1993). Incubations were made in 25 ml glass vials with airtight Teflon-lined screw caps, to avoid losses of the tested compounds. All glassware was acid-washed (HNO_3 10% vol.) and rinsed with acetone and distilled water before the experiments. Physico-chemical conditions of the experiments were 34.20 ± 0.15 ppt salinity, 7.32 ± 0.70 mg l^{-1} O_2 and 8.29 ± 0.11 pH (mean ± std, n = 15).

2.3. Chemical analysis

Test solutions intended for chemical analysis were collected from the experimental vials at the beginning and end (48 h) of the tests. Chemical analyses were conducted with two (high and low) concentrations for each compound. The test solutions were poured into a separatory funnel and PAHs were extracted with dichloromethane (USEPA, 1980). After substitution of the solvent by acetonitrile, the concentration of the PAHs was determined by HPLC with fluorimetric detection. Twenty microlitre of sample were injected into the chromatographic column and a gradient elution was performed by using water and methanol as eluents (López et al., 1996; Viñas, 2002). The fluorimetric detection was carried out by programming the specific excitation and emission wavelengths of each PAH analysed. The recoveries in the extraction method were about 90% for the measured compounds.

2.4. Experimental procedure

The method used in the *M. galloprovincialis* test has been previously described by Bellas et al. (2005). Mature *M. galloprovincialis* were induced to spawn by thermal stimulation in separated beakers with 0.2 μm filtered seawater. 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. A few microlitre of motile sperm were added to the egg suspension and carefully stirred to allow fertilization. Fertilized eggs (ca. 30 eggs/ml) were transferred to vials containing the experimental solutions that were incubated at 18 °C for 48 h, until the second larval stage (D-veliger), characterized by a straight dorsal hinge which gives the larva the shape of a capital letter D, was attained.

P. lividus gametes were obtained by dissection from a single pair of adults according to the methods described by (Beiras and Saco-Álvarez, 2006). Approximately 400 fertilized eggs were delivered into vials with the experimental solutions. The vials were incubated at 18 °C until larvae reached the pluteus stage (approximately 48 h after fertilization), which in this species develops four arms covered by cilia and supported by calcareous skeletal rods.

To test the effects of the selected PAHs on the embryonic development of *C. intestinalis*, 250 fertilized eggs obtained by *in vitro* fertilization following the methods of Bellas et al. (2003), were delivered into experimental vials containing 20 ml of the studied compounds. These vials were incubated in a culture chamber at 18 °C until the tadpole larvae stage is reached (20 h after fertilization). The tadpole larvae consists of a trunk which contain the sensory organs (otolith, ocellus and adhesive papillae) and a tail which contains the notochord.

Concurrently with the experiments conducted in the dark, we carried out bioassays under fluorescent light exposure with mussel, sea-urchin and ascidian larvae, in order to study the potential photoinduced toxicity of PAHs. One set of vials was incubated in darkness and the other set under fluorescent light with a 14:10 h light:dark photoperiod. Cool daylight lamps (Osram L15W/765) were used in order to simulate natural irradiation (emission spectrum range: 380–780 nm; Photosynthetically Active Radiation: 70 μE m^{-2} s^{-1}).

The combined toxicity of the five selected PAHs to *P. lividus* embryos was determined according to the concentration addition (CA) model (Loewe and Muischnek, 1926). CA assumes that all components in the mixture are similarly acting substances sharing identical mechanism of action in the exposed organism. An equitoxic mixture of the studied compounds was used i.e. the ratio of the concentrations of the individual mixture components was kept constant while the total concentration of the mixture was varied. CA can be formulated as

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1,$$

where n is the number of mixture components, c_i is the concentration of the ith component of the mixture, ECx_i is the concentration of the mixture component that induce a x% effect when applied singly. The quotient c_i/ECx_i represent the concentration of the mixture component scaled for its relative toxicity and is usually termed the Toxic Unit (TU) of that component. According to CA, each individual compound in the equitoxic mixture contributes equally to the total toxicity. Due to solubility constraints, EC_{20} rather than EC_{50} values were used to test the mixtures.

After the incubation period mussel, sea-urchin and ascidian larvae were preserved by adding a few drops of 40% buffered formalin, and the percentage of D-veliger and normal tadpole larvae