Aquatic Toxicology 120–121 (2012) 27–34

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

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

Recreating the seawater mixture composition of HOCs in toxicity tests with *Artemia franciscana* by passive dosing

E. Rojo-Nieto[a,*], K.E.C. Smith[b], J.A. Perales[a], P. Mayer[b]

[a] Andalusian Centre of Marine Science and Technology (CACYTMAR), Department of Environmental Technologies, University of Cadiz, 11510 Puerto Real, Spain
[b] Department of Environmental Science, Aarhus University, DK-4000 Roskilde, Denmark

ARTICLE INFO

Article history:
Received 21 February 2012
Received in revised form 9 April 2012
Accepted 11 April 2012

Keywords:
Passive dosing
PAH toxicity
Artemia franciscana
Seawater
Mixtures
Chemical activity

ABSTRACT

The toxicity testing of hydrophobic organic compounds (HOCs) in aquatic media is generally challenging, and this is even more problematic for mixtures. The hydrophobic properties of these compounds make them difficult to dissolve, and subsequently to maintain constant exposure concentrations. Evaporative and sorptive losses are highly compound-specific, which can alter not only total concentrations, but also the proportions between the compounds in the mixture. Therefore, the general aim of this study was to explore the potential of passive dosing for testing the toxicity of a PAH mixture that recreates the mixture composition found in seawater from a coastal area of Spain, the Bay of Algeciras. First, solvent spiking and passive dosing were compared for their suitability to determine the acute toxicity to *Artemia franciscana* nauplii of several PAHs at their respective solubility limits. Second, passive dosing was applied to recreate the seawater mixture composition of PAHs measured in a Spanish monitoring program, to test the toxicity of this mixture at different levels. HPLC analysis was used to confirm the reproducibility of the dissolved exposure concentrations for the individual PAHs and mixtures. This study shows that passive dosing has some important benefits in comparison with solvent spiking for testing HOCs in aquatic media. These include maintaining constant exposure concentrations, leading to higher reproducibility and a relative increase in toxicity. Passive dosing is also able to faithfully reproduce real mixtures of HOCs such as PAHs, in toxicity tests, reproducing both the levels and proportions of the different compounds. This provides a useful approach for studying the toxicity of environmental mixtures of HOCs, both with a view to investigating their toxicity but also for determining safety factors before such mixtures result in detrimental effects.

1. Introduction

Due to their properties and the environmental implications, 7 polycyclic aromatic hydrocarbons (PAHs) are included in the list of Priority Pollutants of the European Water Framework Directive (Council Directive 2008/105/EC, 2008) and 16 PAHs in the United States Environmental Protection Agency (USEPA) Priority Pollutant List (Code of Federal Regulation, 1982). PAHs are characterized by low solubilities in aqueous media, and once dissolved in water they tend to partition to organic phases including living organisms (de Maagd et al., 1998). The levels of PAHs in surface seawater can vary widely. For example, Witt (2002) found concentrations of up to 0.0166 $\mu g\,L^{-1}$ in the Central Baltic Sea (Σ15PAHs). In the Bahia Blanca estuary (Argentina), levels for Σ17PAHs ranged from "undetected" to more than 4 $\mu g\,L^{-1}$ (Arias et al., 2009). In a study of seawater around England and Wales, concentrations as high as 10.7 $\mu g\,L^{-1}$ (Σ15PAHs) were found in unfiltered water (Law et al., 1997). It is not only the heterogeneity in concentrations within a region that results in different exposure, but also the composition of the PAH mixture profile varies. This is rarely addressed in traditional risk assessment and management, which focus on the exposure and effects of single compounds and which might lead to an underestimation of the actual risks (Altenburger and Greco, 2009). It is therefore essential to develop and apply new methods to study the exposure and toxicity of complex mixtures that resemble real cases. Moreover, in field samples it is often of interest to investigate the toxicity of a specific group of chemicals such as PAHs. However, such field samples also contain other pollutants in addition to the target pollutants, which make it practically impossible to determine their toxicity directly using field samples.

The toxicity testing of hydrophobic organic compounds (HOCs) in aquatic media is generally challenging, because the hydrophobic properties of these compounds makes them difficult to dissolve and to maintain constant exposure throughout the test (ECETOC,

* Corresponding author. Tel.: +34 956016587; fax: +34 956 016746.
E-mail address: elisa.rojo@uca.es (E. Rojo-Nieto).

0166-445X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2012.04.006

1996 and OECD, 2000). An additional challenge is to test these substances at the solubility level, while avoiding crystals or micro-droplets of the test substance in the test medium. The conventional dosing technique is spiking at the beginning of the test using a co-solvent, but here the concentrations might still decrease during the test and furthermore the solvent might contribute to or modify the toxicity of the test compounds (Hutchinson et al., 2006). This difficulty in maintaining constant concentrations is even more problematic for mixtures, because compound-specific losses can alter the mixture composition during the test. Passive dosing has recently been introduced for establishing and controlling exposure levels by equilibrium partitioning from a dominating donor phase (Bandow et al., 2009; Brown et al., 2001; Kiparissis et al., 2003; Mayer et al., 1999; Smith et al., 2010a). A key feature of passive dosing is that it allows freely dissolved concentrations and chemical activities to be controlled throughout the test. It has recently been proposed, experimentally confirmed, that chemical activity is a key parameter for the baseline toxicity of hydrophobic organic chemicals, and that some highly hydrophobic substances can exert baseline toxicity at very low aqueous concentrations but at relatively high chemical activities (Engraff et al., 2011; Mackay et al., 2009, 2011; Mayer and Holmstrup, 2008; Mayer and Reichenberg, 2006; Reichenberg and Mayer, 2006; Smith et al., 2010a). Diffusion across membranes occurs spontaneously down chemical activity rather than concentration gradients, with equilibrium partitioning being defined by equal chemical activities (Mayer and Reichenberg, 2006). Chemical activity is not related to total concentrations, but with the freely dissolved concentration (C_{free}), which can be understood as the concentration of freely dissolved molecules (not sorbed or bound to other phases).

The first aim of this study was to compare co-solvent spiking and passive dosing in a marine toxicity test with *Artemia franciscana* Kellogg (1906) (brine shrimp). These experiments were done in parallel and under identical conditions for the optimal comparison of the two dosing techniques. The second aim was to determine the toxicity of 10 PAHs to brine shrimp at exactly their respective solubility limits, and to compare the results to other tested organisms. The third aim was to explore the potential of passive dosing for recreating the composition of an environmental HOC mixture in an aquatic toxicity test.

2. Materials and methods

2.1. Chemicals and materials

Naphthalene (99%, Aldrich, Germany), acenaphthene (99%, Fluka, Germany), fluorene (99%, Fluka), phenanthrene (>99.5%, Aldrich), anthracene (99%, Fluka), pyrene (>99%, Sigma, Germany), fluoranthene (99%, Aldrich), benz[a]anthracene (99%, Aldrich), benzo(a)pyrene (98%, Cerilliant, TX, USA) and chrysene (99%, Cerilliant) were used. Ten milliliter autosampler vials with Teflon lined screw caps were bought from Mikrolab (Aarhus, Denmark). Medical grade PDMS silicone was made using the MDX4-4210 kit from Dow Corning, supplied by the Institute of Anaplastology (Velten, Germany). Lint-free lens tissue was used for cleaning (Bie and Bernsten A/S, Denmark). Methanol (HPLC grade) was used for extraction and analysis (Merck, Darmstatd, Germany) and MilliQ water (Super Q treated, Millipore, MA, USA) for rinsing.

2.2. Artificial seawater

Artificial seawater was prepared by dissolving NaCl (21.03 g L^{-1}), Na_2SO_4 (3.52 g L^{-1}), KCl (0.61 g L^{-1}), KBr (0.088 g L^{-1}), $Na_2B_4O_7 \times 10H_2O$ (0.034 g L^{-1}), $MgCl_2 \times 6H_2O$ (9.5 g L^{-1}), $CaCl_2 \times 2H_2O$ (1.32 g L^{-1}), $SrCl_2 \times 6H_2O$ (0.02 g L^{-1}), and $NaHCO_3$ (0.17 g L^{-1}) in Millipore water as recommended by the USEPA for acute toxicity tests with marine organisms (USEPA, 2002). The final total salt concentration was 0.45 M.

2.3. Artemia franciscana nauplii individuals for toxicity tests

Toxicity assays were carried out using a homogeneous *A. franciscana* population that had been incubated in the same medium and grown synchronously. Certified cysts of *A. franciscana* nauplii (AF450, INVE) were bought from Acuazul, S.C. (Spain). Cysts were hatched at 20 °C in artificial seawater and under artificial light illumination with a light/dark period of 16/8 h. Hatching was carried out under conditions that were as similar as possible to conditions in the toxicity tests. The only parameter that was changed between the hatching procedure and toxicity assays was light, since according to the Food and Agriculture Organization of the United Nations (FAO) (Sorgeloos et al., 1986) illumination is essential for optimal hatching of *Artemia* cysts, at least for the first hours after hydration. Newborn nauplii were isolated under a stereoscopic microscope with a Pasteur pipette, and 10 nauplii of less than 5-h-old added to each passive dosing or spiking vial.

As has been suggested by several authors (Hadjispyrou et al., 2001; Koutsaftis and Aoyama, 2007; Nunes et al., 2006; Sarabia et al., 2002) there are a number of reasons for the selection of brine shrimp, *Artemia* sp., as a test organism. It is gaining popularity as a test organism because of the continuous availability of *Artemia* in the form of dormant eggs (cyst), and its adaptability to wide ranges of salinity and temperature. Since test animals hatching from cysts are of a similar age, genotype and physiological condition the test variability is greatly reduced, guaranteeing reliability, feasibility and cost effectiveness in routine and/or research ecotoxicity tests. For all these reasons, *Artemia*, or more precisely *A. francisacana*, has been chosen as test organism for the acute assays in artificial sea water in this study. The chosen end point was mortality of the recently (instar II–III stage) hatched nauplii, a well-accepted end point in cyst-based toxicity assays with *Artemia* (Nunes et al., 2006).

2.4. Preparation and loading of the passive dosing vials

The PDMS silicone pre-polymer and catalyst were mixed according to the supplier, and 500 mg (±1%) were cast into the base of the 10 mL autosampler vials. These were then stored overnight at 4 °C to allow any air bubbles formed during casting to escape, before placing in an oven at 110 °C for 48 h to cure. The cured silicone was rinsed 3 times with methanol to remove oligomers and other impurities, followed by 3 rinses with MilliQ water to remove any adhering methanol. Finally, the surfaces were dried with lint-free tissue.

For the single PAH toxicity assays and salting-out experiments (conducted to know the exact solubility of PAHs in this specific artificial seawater), passive dosing vials were loaded to saturation with individual PAHs using methanol suspensions of each compound (Smith et al., 2010a). The PAH crystals in the methanol suspension served to maintain the saturation level in the methanol and thus also in the silicone. For the PAH mixture toxicity assays, the PDMS silicone in the vials was loaded to below saturation using mixtures of the PAHS dissolved in methanol at the appropriate concentrations. The volume of methanol PAH mixture solution used was sufficient to ensure negligible depletion during the loading process. The PAH mixture profile was taken from an unfiltered superficial sea water sample, collected as part of a monitoring program from a coastal area of Spain, in the Bay of Algeciras.

Measured values of the PAH equilibrium partitioning ratios between PDMS silicone and the seawater used in this study,