Aquatic Toxicology 147 (2014) 32–40

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Aqueous phenanthrene toxicity after high-frequency ultrasound degradation

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ARTICLE INFO

Article history:
Received 9 October 2013
Received in revised form
22 November 2013
Accepted 8 December 2013

Keywords:
Cytotoxicity
Genotoxicity
Hemocytes
Phenanthrene
Ultrasonic treatment

ABSTRACT

Given that polycyclic aromatic hydrocarbons (PAHs), such as phenanthrene (PH), possess a potent risk for aquatic biota, a great attempt to develop and apply advanced oxidation processes, such as ultrasound (US), is of great concern nowadays. However, because US PAH-derived toxic intermediates are difficult to detect, the present study investigates aqueous PH toxicity before and after high-frequency US degradation, in hemocytes of mussel *Mytilus galloprovincialis*. Specifically, cell viability (with the use of neutral red uptake/NRU method), and oxidative-stress indices in terms of superoxide anions, ($^{\bullet}O_2^-$), nitric oxides (NO, in terms of nitrites), lipid peroxidation products (in terms of malondialdehyde/MDA content) and DNA damage (with the use of Comet assay method) were investigated in mussel hemocytes exposed to environmentally relevant concentrations of PH (0.01, 0.1, 1 and 10 $\mu g\,L^{-1}$), before and after US treatment for 120 min (at a frequency of 582 kHz). According to the results, the NRU method showed a significant attenuation of PH-induced mortality in US PH-treated hemocytes in all cases. Moreover, the increased levels of $^{\bullet}O_2^-$ and NO generation, as well as MDA content measured in PH-treated hemocytes, were drastically decreased after US degradation in any case. Similarly, the disturbance of DNA integrity (in terms of % DNA in tail, OM and TM), was negligible in case of US PH-treated hemocytes. Although further *in vitro* and *in vivo* studies are needed, the present study showed for the first time that high frequency US could be applied as a highly efficient and "environmentally friendly" process for degrading low molecular weight PAH, such as PH.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are present in various environmental systems including coastal estuaries and marine sediments (Daskalakis and O'Connor, 1995) as well as in drinking water supplies (Kim Oahn et al., 2002). Direct discharges into the marine environment from point sources, such as wastewater treatment plants, range from <1 $\mu g\,L^{-1}$ to over 625 $\mu g\,L^{-1}$, whilst concentrations of PAHs in industrial effluents range from undetectable to 4.4 $mg\,L^{-1}$ (Latimer and Zheng, 2003). Their presence, even at low concentrations, could be toxic for aquatic biota, since due to their hydrophobicity they can be easily taken up by marine organisms, thus binding to lipophilic sites of cellular molecules (Camus et al., 2002). This PAH accumulation pattern seems to be a feature of bivalves, such as mussels and oysters, which have been reported to accumulate chiefly, and rapidly, PAHs of low molecular weight, such as phenanthrene (PH), because of their higher water solubility (Orbea and Cajaraville, 2006). In particular, PH is able to readily accumulate in marine biota, in concentrations that are dependent merely upon the ability of species to biotransform them (Guinan et al., 2001).

During the last decade, a wide variety in the types and magnitudes of physiological responses, which determine PAHs bioavailability and toxic potency in marine organisms, such as bivalve mollusks, has been reported by a lot of studies (Galgani et al., 2011; Giannapas et al., 2012; Grintzalis et al., 2012; Hannam et al., 2010). According to the latter, as well as taking into account that the natural degradation process of PAHs is very slow and they persist in the environment for a long period of time (Cerniglia, 1992), there is a great attempt to develop effective and economical treatment methodologies.

The widely used biological treatment processes have a lot of disadvantages, mainly related to (a) their inhibition due to the presence of toxic or persistent pollutants, (b) their sensitivity to several

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http://dx.doi.org/10.1016/j.aquatox.2013.12.006

environmental factors, as well as (c) their high-cost, especially when they are performed in the presence of high or low concentrations of micropollutants (for a review see Adewuyi, 2005). On the other hand, during the last decade, advanced oxidation processes have received increasing attention for the destruction of various organic pollutants commonly found in waters and wastewaters (Psillakis et al., 2004). Among them, ultrasound (US) treatment methodologies have been employed as emerging advanced oxidation processes for a wide variety of micropollutants, including PAHs (David, 2009; Kim et al., 2001; Lifka et al., 2003; Manariotis et al., 2011; Matouq et al., 2008; Sponza and Oztekin, 2010; Virkutyte and Rokhina, 2010). In fact, the US treatment methodologies are of great concern, since they do not require the use of additional chemicals (*e.g.* oxidants and catalysts) commonly employed in several oxidation processes, thus avoiding the respective costs as well as the need to remove the excess of toxic compounds prior to discharge (Psillakis et al., 2004; Sponza and Oztekin, 2010).

Despite the effectiveness of the US treatment process, the production of toxic intermediates during PAH degradation remains still unclear due to their low and undetectable concentration (Manariotis et al., 2011). Specifically, it has been reported that US PAHs intermediate products (hexane-extractable metabolites determined by liquid–liquid extraction and GC–MS) were not detected, probably due to their presence at concentrations lower than the detection limit ($\approx 1\,\mu g\,L^{-1}$). Since the production of undetectable intermediates with toxic potency could not be excluded, further studies including the determination of their biological effects both under *in vivo* and *in vitro* conditions are needed.

Marine bivalve mollusks, such as the mussel *Mytilus galloprovincialis*, are widely used for predicting the effects of xenobiotic compounds being present into the water (for a review see Dailianis, 2011). In specific, mussel hemolymph is considered as a transfer medium for xenobiotics, while hemocytes' viability and function integrity could affect immunosurveillance and the concomitant health status of the organism (Alvarez and Friedl, 1992; Matozzo et al., 2001). Since hemocytes are susceptible to the deleterious effects of xenobiotic and chemical compounds (Pan et al., 2006), primary cell cultures of mussel hemocytes have been increasingly used for estimating the effects of toxic compounds with great precision and reproducibility (Banakou and Dailianis, 2010; Bouki et al., 2013; Cao et al., 2003; Chatziargyriou and Dailianis, 2010; Dailianis, 2009; Dailianis et al., 2009; Olabarrieta et al., 2001; Patetsini et al., 2013; Tsiaka et al., 2013; Vouras and Dailianis, 2012).

Given that the development of PAH degradation technologies is of great interest, but the toxic effects of PAH-derived intermediates remains still scarce, the aim of the present study was to investigate the toxic effects of PH on mixed primary hemocytes of mussels, before and after treatment with high-frequency US. In specific, considering PAHs-mediated cytotoxic and oxidative effects (Giannapas et al., 2012; Grintzalis et al., 2012; Hannam et al., 2010), a battery of stress indices was investigated in mussel hemocytes exposed *in vitro* to PH and US PH-treated aliquots (possibly include PH-derived intermediates), such as cell viability (in terms of neutral red uptake/NRU assay), oxidative-stress indices, in terms of superoxide anions ($^{\bullet}O_2^-$), nitric oxides (NO, in terms of nitrites) and lipid peroxidation products (in terms of malondialdehyde/MDA content). Furthermore, since PAHs, such as PH, as well as their metabolic products may disturb DNA integrity (Altenburger et al., 2003), DNA damage was determined with the use of alkaline single-cell gel electrophoresis (Comet assay). Overall, an integrated approach is provided, regarding the toxic effects of PH and its intermediates, possibly occurring after US degradation process.

2. Materials and methods

2.1. Chemicals and reagents

All reagents and solvents used were of the highest analytical grade and purity (for more details see SM 2.1).

2.2. Ultrasound PH degradation test method

2.2.1. Preparation of PH stock solution

Phenanthrene was dissolved in acetone at a concentration of $0.3\,g\,L^{-1}$ (stock solution). An appropriate quantity of the PH stock solution (0.2 mL) was diluted with double distilled water (final volume 1 L), in order to yield a stock solution of PH at concentration of $\approx 60\,\mu g\,L^{-1}$, which was kept in the dark at 4 °C, before being used for further analysis. Final concentration of acetone in the aqueous stock solution was 0.02% (v/v). Aqueous PH concentrations were measured by a cuvette mode fluorescence spectrophotometer (Cary Eclipse, Varian Australia Pty Ltd., Australia) using a quartz cuvette and excitation/emission wavelength of 249/347 in triplicate. The PH concentration in the aqueous solution was determined based on a standard calibration curve (see SM Fig. 1).

2.2.2. PH-degradation by ultrasound

An ultrasonic system (Meinhardt Ultraschalltechnik, Leipzig, Germany) composed of a 75-mm diameter titanium transducer operating at 582 kHz, a function generator, and an amplifier were employed in this study, based on the method described by Manariotis et al. (2011). The electric power of the system was adjusted at 133 W. The transducer was mounted at the bottom of a cylindrical 2-L glass laboratory reactor with double walls to allow water circulation for cooling. Aqueous solutions of PH (1000 mL) were poured into the ultrasonic reactor. A sample solution was taken to measure and record the initial PH concentration. During each experiment, 4 mL samples were taken at 10, 20, 30, 60, 90 and 120 min with a glass pipette and stored in glass vials before analysis. All samples were analyzed at the end of each experiment and the PH concentrations were compared to initial concentration (see SM Table 1). The initial concentration of the solution was $61 \pm 4.3\,\mu g\,L^{-1}$ (3 independent experiments in each case). The value is 102% the calculated value. The temperature of the reactor liquid was measured with a digital thermometer equipped with a thermistor (Oakton Temp 5 Acorn Series, Eutech Instruments Ltd., Singapore).

2.3. Mussel collection and handling

The Mediterranean mussel *M. galloprovincialis* (Lmk. 1819) is common and not endangered invertebrate species. Since no permits were required for their use in both *in vitro* and *in vivo* studies, the experimental procedure (in terms of acclimation period, mussel handling and exposure procedure) was appropriately carried out in order to minimize animal suffering. In brief, mussels (4–5 cm long) were collected from a government-certified fish-mussel farm located to the north side of Korinthiakos Gulf (Gulf of Kontinova, Galaxidi, Greece), which is characterized by negligible levels of inorganic and organic pollution (Giannapas et al., 2012; Kalpaxis et al., 2004), transferred and acclimated under laboratory conditions for 7 days in static tanks, containing aerated (dissolved oxygen 7–8 $mg\,L^{-1}$ at 15 °C and 35‰ salinity, 14:10 h light:dark photoperiod), recirculated UV-sterilized and filtered artificial sea water (ASW). Mussels were maintained without feeding during the acclimation period and then fed daily (approximately 30 mg of dry-microencapsules, Myspat, Inve Aquaculture^{NV}, Belgium/mussel).