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Environmental fate and ecological risks of nonylphenols and bisphenol A in the Cape D'Aguilar Marine Reserve, Hong Kong

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ABSTRACT

Nonylphenols (NPs) and bisphenol A (BPA) are the most common endocrine disruptors detected in the coastal waters of Hong Kong. The Cape D'Aguilar Marine Reserve (CAMR), the only marine reserve in Hong Kong is close to urbanized areas, thus the resident marine organisms are inevitably influenced by partially treated wastewater from adjacent sewage treatment plants (STPs). Elevated levels of NPs and BPA were detected in all seawater, sediment and biota samples collected from the CAMR. Estrogenic activities of seawater from the CAMR, and sludge and sewage from a nearby STP were assessed using yeast estrogen screen assay. We found aromatase, estrogen receptor and vitellogenin genes in the marine medaka fish *Oryzias melastigma* were significantly up-regulated after exposure to the reserve's seawater. According to a tissue-residue-based probabilistic risk assessment, the marine species living in the CAMR are having 35% and 21% of chance to be at risk due to exposure to NPs and BPA, respectively.

1. Introduction

Cape D'Aguilar is situated on the southeastern tip of Hong Kong Island in the Hong Kong Special Administrative Region (SAR) of China. In 1996, the Hong Kong SAR Government, in recognition of the importance and urgency of protecting the geomorphological and ecological environment of Cape D'Aguilar, designated this area as the first and, to date, only marine reserve in Hong Kong (Fig. 1). The marine reserve has a rich marine biodiversity, with numerous habitats such as intertidal sea arches and subtidal caves (zawns), hermatypic and ahermatypic coral reefs, exposed and sheltered rocky beaches, cobble beaches, intertidal pools of varying dimensions and elevations and protected under-boulder landscapes, resulting in an associated rich marine biodiversity (Morton and Harper, 1995). Owning to its small size (0.2 km^2) and location close to sources of human activities, the marine reserve is considered vulnerable to chemical contaminants from point sources such as nearby sewage treatment plants (STPs) (Xu et al., 2014, 2015). These discharges were identified as major vectors for the entry of toxic and endocrine disrupting chemicals (EDCs) such as nonylphenols (NPs) and nonylphenol ethoxylates (NPEOs) into receiving waters in Hong Kong (Kueh and Lam, 2008). Li et al. (2007) discovered that concentrations of NPs ranged from 29 to 2591 ng/L in surface estuarine water samples collected from the Mai Po Marshes Nature Reserve in northwestern Hong Kong. There are increasing concerns over the adverse effects that may result from the exposure of wildlife to these EDCs. Numerous laboratory experiments indicate that the EDCs may cause negative health effects (e.g. growth, behaviour, reproduction and immune function) in fishes through disrupting their endocrine systems (e.g. Länge et al., 2001; Marta and Charles, 2012).

Of particular concerns are NPs and bisphenol A (BPA), which have been identified as major anthropogenic contributors to endocrine-disrupting activities in aquatic environments (Auriol et al., 2006). NPs are the main degradation products of alkylphenol polyethoxylates, which have been used widely as surfactants in household detergents, agriculture and the dyeing industry (White et al., 1994). NPs can cause an increase of vitellogenin mRNA and a decrease in the growth rate of testes in male rainbow trout at a concentration of 1 μg/L (Lech et al., 1996; Bonefeld-Jørgensen et al., 2007). BPA is an industrial raw material mainly used in plastic, rubber, adhesive, and cable industries, and is known to cause a delay in the hatching of eggs and a suppression of growth in juvenile rainbow trout (Aluru et al., 2010; Bonefeld-Jørgensen et al., 2007). Due to concerns about the possible impacts of NPs and BPA on aquatic organisms, the levels of these chemicals have been quantified in different aquatic systems around the world with

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concentrations ranging from a few ng/L to hundreds μg/L (Isobe et al., 2001; Mayer et al., 2007; Lee et al., 2013). In parallel with chemical analysis, *in vivo* and *in vitro* bioassays have been applied to assess the integral estrogenic activity in environmental samples (Sonneveld et al., 2006). Estrogenic activities derived from bioassays have been reported upon in different environmental matrices from coastal marine environments of various countries (Ra et al., 2011; Cargouet et al., 2004; Vermeirssen et al., 2005).

In a previous study, we found that effluents from STPs in Hong Kong contained significant concentrations of varying EDCs, with NPs and BPA being the most abundant (Xu et al., 2014). Little is known, however, about the occurrence and distribution of the two compounds in the coastal waters of Hong Kong. Cape D'Aguilar Marine Reserve (CAMR) is an important habitat and spawning ground for a highly diverse assemblage of marine organisms, but there are no data concerned with the bioaccumulation of EDCs in the various species the reserve protects. Despite the previous detection of NPs and BPA at hundreds of ng/L in waters collected from the marine reserve, overall estrogenic activities in the waters and sediments of the marine reserve remain unknown.

The objectives of this study were: (1) to investigate the occurrence of NPs and BPA in Hong Kong's coastal waters; (2) to analyse the concentrations of NPs and BPA in surface seawater, sediment, and tissues of eleven marine organisms sampled from the CAMR in both the wet (summer) and dry (winter) seasons and (3) to assess the estrogenic activities of NPs, BPA and their admixture at environmentally realistic concentrations, as well as different environmental samples collected from the CAMR using *in vitro* yeast estrogen screen (YES) assay and *in vivo* mRNA assays with the marine medaka fish *Oryzias melastigma*. This study provides a better understanding of the occurrence and ecological risks of EDCs in the Hong Kong coastal waters and, in particular, the Cape D'Aguilar Marine Reserve.

2. Materials and methods

2.1. Sampling

Surface seawater samples were collected from the east, south and west coasts of Hong Kong in August and October 2012 (Fig. 1). Seawater, sediment, and biota samples (i.e., of the algae, snails, mussels, sea urchins, sea cucumbers, shrimps, crabs and fishes) were collected from the CAMR in January and August 2013 (see Fig. 1). Water samples were taken from 1.5 m below the surface. About 50 ml of methanol was immediately added to 1 L water sample to suppress biological activities and the pH adjusted to 3–4 using 4 M H_2SO_4 on the research boat *SWIRE Asterina*. Water samples were transported on ice to the laboratory and stored at 4 °C in darkness. The samples were subjected to chemical analysis within 24 h. Surface sediment samples (up to the top 20 cm) were collected using a stainless steel grab sampler, scooped into glass jars and stored at −20 °C until analysis. Wastewater and sludge samples were collected from the Marine Reserve STP in August 2013. All sediment tissue and sludge samples were freeze-dried and stored at −20°C until analysis.

2.2. Water quality measurement in the CAMR

A Water Quality Monitoring Buoy System (EMM68, Yellow Springs, USA) equipped with sensors for measuring water temperature, salinity, dissolved oxygen, conductivity, pH, turbidity, chlorophyll *a* and phycocyanin was deployed in the CAMR (see Fig. 1). The results of the monitored water quality parameters during the sampling time (January and August 2013) are given in Table S1 (Appendix A).

2.3. Chemical analysis

The analytical procedure for the NPs and BPA was based on Zhao et al. (2011). Briefly, 1 L each of the influent, effluent and seawater samples was filtered through a glass fibre filter (Whatman GF/F, 0.7 μm, UK). Methanol was used to elute non-filterable particles on the filter and these were combined with the filtered sample. For solid phase extraction (SPE), 100 μL of 1 mg/L of 4-n-NP was added to each sample and served as internal standards. SPE cartridges (Oasis HLB, 6 mL, 500 mg) were preconditioned with methanol/HPLC water (1:1, v/v). The filtered water samples were passed through the SPE cartridges at 10 mL/min. The target compounds were eluted from the cartridges using methanol and dichloromethane. The extracts were then dried and re-dissolved in methanol. Target compounds in the sediment, sludge, or tissue samples were extracted by ultrasonic-assisted solvent extraction. Five grams of the prepared samples were mixed with ethyl acetate (10 mL). The tube was ultrasonicated for 15 min and centrifuged at 1370*g* for 10 min, and the supernatant collected. The sediments were extracted twice more with 10 mL and 5 mL of ethyl acetate, respectively. The supernatants were combined and concentrated to about 1 mL on a rotary evaporator. The extract was further purified with a glass column loaded with 1 g of silica gel. Elution was carried out using ethyl acetate. The eluted sample was concentrated to near dryness under a gentle nitrogen stream and re-dissolved in 1 mL methanol for further treatment. For derivatization, 100 μL of an extract was removed to a tube and dried. Prior to 2 mL of 1 M $NaHCO_3$ solution and 1 mL of 1 M NaOH solution being added into the sample, 2 mL of n-hexane, 50 μL of 10% pyridine in toluene and 50 μL of 2% PFBOCl in toluene were added in sequence. After separation, the supernatant of the n-hexane phase was transferred to a vial and dried. The final extract was re-dissolved in hexane for gas chromatography-mass spectrometry (GC–MS) analysis or in DMSO for yeast estrogen screen (YES) assay. The target compounds were separated by the GC (Agilent 6890N) with a DB-5MS capillary column (length: 30 m; i.d.: 0.25 mm; coated film thickness: 0.25 mm). The MS (Agilent 5973) was used as the detector and operated in the selected-ion monitoring mode with electron-impact ionization (ionization voltage, 70 eV). The oven temperature programme was as follows: the initial temperature was 70 °C for 1 min, then increased to 170 °C at 20 °C/min, to 230°C at 6 °C/min, to 280 °C at 12 °C/min for 6 min, and held at 300 °C for 2 min. The injector was set at 280 °C. The GC–MS interface and the ion-source temperatures were at 280 °C and 250 °C, respectively. Helium was used as the carrier gas at 1 mL/min. A 1 μL sample was injected in splitless mode with a solvent delay of 3 min. The characteristic ions and retention times of the target compounds were obtained and identified with full scan mass spectra from m/z 50 to 500, the compounds of interest being identified in SIM mode.

2.4. Yeast estrogen screen (YES) assay

YES bioassay was conducted according to Ma et al. (2005). The recombinant yeast (*Saccharomyces cerevisiae*) cells were constructed by Brunel University, U.K. (Routledge and Sumpter, 1996) and kindly provided by Dr. Mei Ma from the State Key Laboratory of Environmental Aquatic Chemistry, Chinese Academy of Science, Beijing, China. The yeast strain was grown at 30 °C, 130 rpm overnight, in Erlenmeyer flasks using $CuSO_4^-$ supplemented (10^{-8} M) SC medium. In performing the assay, exponentially growing overnight cultures were diluted with the SC medium to an OD at 600 nm of 0.25; the absorbance was measured using a spectrophotometer (SpectroMax M2e, Molecular Devices, USA). All environmental samples were assayed with a minimum of three replicates. Each sample assayed included positive

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