Relative Contribution of Endocrine Disrupting Chemicals to the Estrogenic Potency of Marine Sediments of Osaka Bay, Japan

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Abstract

Although concentrations of endocrine disrupting chemicals (EDCs) in seawaters of Japan are relatively low, vitellogenin and ovo-testis inductions are still being observed in some males of mullet and flounder collected in coastal areas. These fish species are benthic and could be affected by EDCs in marine sediments. Therefore, the concentrations of EDCs in marine sediments of Osaka Bay were determined by LCMS/MS. In addition, the estrogen receptor binding potencies as the estrogenic potencies of these sediments were assessed by the medaka estrogen receptor-α binding assay. Results show that estrogenic potencies were higher in sediments of the inner part of the bay especially at station 13 (off Sakai City) where quite strong estrogenic potency was detected. Through calculation of total E2 equivalent concentration in sediments, it was established that approximately 50% of estrogenic potency was due to nonylphenol, estrone and 17β-estradiol suggesting that these compounds play important roles as endocrine disruptors in coastal environments of Osaka Bay.

Keywords: estrogen disrupting chemicals, E2 equivalent concentration, marine sediments, Osaka Bay

1. Introduction

Endocrine disrupting chemicals (EDCs) are widely known to act like hormones in animals and sometimes adversely affecting their health. For instance, several reports show that many EDCs affect fish reproduction including the induction of vitellogenin in males rainbow trout (Jobling et al., 1996), and grey mullet (Aoki et al., 2010) and Japanese common goby in Osaka Bay (Ohkubo et al., 2003). Most studies focus on EDCs in water but the detected concentrations are too low to cause adverse effects on fish reproduction except perhaps for waters impacted by sewage effluents (Desbrow et al., 1998; Routledge et al., 1998; Vajda et al., 2008; Japanese Ministry of Environment, 2002). The relative hydrophobic characteristics of representative EDCs such as natural hormones, however, suggest potential abundance of EDCs in sediments. Even the ovo-testis inductions in some marine benthic fishes (Wanami et al., 2003) support speculations that marine sediments are reservoirs of EDCs. As such, some researchers have already reported detecting higher concentration of EDCs in freshwater and marine sediments (Khim et al., 1999; Isobe et al., 2001; Braga et al., 2005; Isobe et al., 2006). It has been suggested also that EDCs in sediments could be accumulated by fish or other aquatic organisms through the benthic food chain (Mayer et al., 2007; Vigano et al., 2006; Kono et al., 2008; Kono et al., 2010).

Anthropogenic EDCs such as nonylphenol (NP), octylphenol (OP) and bisphenol A (BP) and natural estrogens such as 17β-estradiol (E2) and estrone (E1) are already detected at relatively high concentrations in marine sediments (Khim et al., 1999; Isobe et al., 2001; Braga et al., 2005; Isobe et al., 2006). These chemicals affect fish by competitively binding with the estrogen receptors. To assess the threat posed by sediments as reservoirs of EDCs, estrogenic potency of sediment extracts should be evaluated. However, reports of total estrogenic potency of sediments in relation to EDC residues are rare. To evaluate EDCs in sediments for marine benthic fish, estrogen receptor of marine fish must be naturally applied,

however only medaka estrogen receptor-α can be applied in our laboratory. Therefore, in the present study, a competitive ligand binding assay using medaka estrogen receptor-α (medaka ER) was applied to evaluate estrogenic potency of sediments of Osaka Bay. Medaka ER binding assay, as a mechanism-based assay, is a useful tool in assessing environmental risks posed by complex samples such as sediments and sediment extracts that sometimes show cytotoxicity (Khim et al., 1999; Keiter et al., 2006). Concentrations of target EDCs in the sediments were also determined to evaluate specific contribution of each EDC potency vis a vis the total estrogenic potency of sediment extracts.

2. Materials and methods

2.1. Collection and preparation of sediments

Osaka Bay, which covers 1500 km^2, is one of the biggest inner bays in Japan. However, with 6 million people living near it and 103 municipal wastewater treatment plants around the area, it is also one of most polluted bays in the country. Sediments were collected by Smith- McIntyre grab in 28 sites (shown in **Fig.1**) around the bay in 2000 and 2001 on-board the Shirafuji-maru, a research vessel of the National Research Institute of Fisheries and Environment of Inland Sea. Upon reaching the deck, sediment samples from 0 to 10 cm deep were collected by stainless spatula and immediately frozen at -20°C for further analysis.

After thawing and sieving of sediments in stainless sieves, particles less than 2 mm were dried at 60°C for 24 hours and kept in glass bottle until analysis. Dried sediments were subjected to further sieving and total organic carbon content (TOC) was determined in particles that pass through a 62.5-μm sieve.

2.2. Determination of EDCs

About 5 g of dry sediment was subjected to ultrasonic extraction twice with 20 mL

of dichloromethane-methanol (3:1, v/v) for 20 min. After centrifugation at 200 rpm for 10 min, extract was concentrated to 0.5 mL by rotary evaporator (rotavap) and gentle blow of nitrogen (N2) gas. Concentrated extracts were added with 4.5 mL of 0.05M sodium acetate buffer before loading unto 200-mg Strata X-AW cartridges (Phenomenex, Macclesfield, UK), which were previously conditioned with 5 mL of ethyl acetate, 5 mL methanol, and 20 mL distilled water in sequence. The target chemicals were eluted with 10 mL ethyl acetate after washing the cartridge with 4 mL 0.05M sodium acetate buffer-methanol (9:1, v/v). The eluate was dried using N2 gas and the residue added with 0.5 mL cyclohexane-ethyl acetate. The solution was loaded to Sep-pak silica cartridge (Waters Co., Massachusetts, USA) previously conditioned with 4 mL cyclohexane-ethyl acetate (6:4, v/v) and 4 mL cyclohexane. The target chemicals were eluted again with 10 mL cyclohexane-ethyl acetate (6:4, v/v) after washing the cartridge with 4 mL of cyclohexane. The 17β-estradiol-16,16,17-d_3, as internal standard, was added to the eluate followed by drying under gentle blow of N2 gas. Exactly 200 μL acetonitrile was added to the dried residue for determination by LCMS/MS.

LCMS/MS analysis was carried out with an Agilent 1200 LC system (Agilent Technologies, California, USA) coupled to an API 200 triple stage quadruple mass spectrometer equipped with ESI source (Applied Biosystems, California, USA). The optimized parameters were set as follows: curtain gas (40 psi), turbo gas (80 psi) and auxiliary gas (80 psi) using nitrogen; CAD gas, 5 psi; ion voltage, -4500V; and turbo temperature, 400°C. Ionization and fragmentation settings were optimized by direct injection of the solution containing target chemicals.

EDCs were individually separated by the gradient liquid chromatography system using column of ZORBAX Extend-C18 (2.1 mm i.d. × 100 mm, 3.5 μm of particle size, Agilent) in 40°C, and the injection volume was 10 μL. Gradient elution with a flow of 200 μL/min and acetonitrile (solvent A) and 0.1M triethyl amine (v/v, solvent B) was carried out

using the following conditions: held 20% A in 2 min from starting, increased to 95% A at 2 to 15 min, and held to 22 min; and decreased 20% A to 22.1 min, and held to 32 min. The negative ion multiple reaction monitoring (MRM) mode was used for the transition of NP (m/z: 219.0-132.9), E2 (m/z: 271.1-144.9), BP (m/z: 227.1-211.8), OP (m/z: 205-132.9), E1 (m/z: 269.1-145.0) and E2-d_3 (m/z: 274.2-144.9).

Detection limits of NP, E1, BP, OP and E2 were 0.4, 0.2, 1.0, 0.4 and 1.0 ng/g dry weight (d.w.), respectively. On one hand, the recovery rates of NP, OP, BP, E1 and E2 in the sediment were 98, 76.4, 95.6, 133 and 119%, respectively. Determinations were replicated more than 3 times.

2.3. Medaka ER binding assay

A 1 g of dried sediment was subjected to extraction by ultrasonication twice using 10 mL dichloromethane and once using 10 mL methanol. Extracts were combined and centrifuged at 430 g (4°C for 10 min) before concentration using rotavap and N2 gas. The solvent was ultimately replaced with 1 mL dimethylsulfoxide (DMSO). The DMSO solutions were applied to medaka ER-α competitive binding assay (Chemical Evaluation and Research Institute, Japan) following Nakai (Nakai, 2003; Nakai et al., 2004) with slight modifications. Briefly, 10 μL sample solution and10 μL 5 nM [2,4,6,7,16,17-^{3}H] 17β-estradiol were dissolved in Tris-HCl (pH 7.4, 70 μL) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/mL albumin, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin and 1mM dithiothreitol. After incubation for 1 hour at 25°C, 100 μL dextran-coated charcoal (DCC, 0.2% activated charcoal and 0.02% dextran in 7.4-pH PBS) was added and incubated again for 10 min at 4°C to remove the free radioligands. After centrifugation, radioactivity in supernatant was measured by liquid scintillation counter. The estrogenic potency of sediment extracts were determined by the inhibition rates of binding of ^{3}H-labeled 17β-estradiol to medaka ER.

Similarly, inhibition rates of binding of ^{3}H-labeled 17β-estradiol to medaka ER due to competitive binding by target EDCs (i.e., NP, OP, BP, E1 and E2) were examined to find the median inhibition concentration (IC_{50}). All analyses were conducted with more than 2 replicates.

2.4. Estimation of estrogenic potency

After the medaka ER binding assay, IC_{50}s of the target EDCs (NP, OP, BP, E1 and E2) were determined by probit analysis using SPSS (Windows Ver. 14.0J, SPSS Inc., USA). The *relative E2 potency* (REP) of each EDC was calculated by the ratio of IC_{50}s of E2 and of a particular EDC. On the other hand, *E2 equivalent concentration* (EEQ) of each EDC in the sediment extract was estimated by multiplying the detected concentration of a particular EDC in the sediment and its REP. EEQ of all EDCs were summed up to get the *total EEQ*. Using the total EEQ, estimates of probable inhibition rate due to EDCs were calculated. Finally, the percentage contribution of EDCs to the estrogenic potency of sediment extracts was assessed by comparing the measured and estimated inhibition rates.

2.5. Spatial distributions

Spatial distributions of estrogenic potency and target EDC concentrations allover Osaka Bay were simulated by GIS software (Marine Explorer, Environmental Simulation Laboratory Co. Ltd., Japan).

3. Results and discussion

3.1. Concentrations of target EDCs

The ranges of NP, OP, BP, E1 and E2 in the sediments were ND (<0.1 ng/g d.w.)-119, ND-15.6, ND-6.8, ND-0.9 and ND-1.57 ng/g d.w., respectively. E2 was detected only at

stations 8, 13 and 21 with the highest concentration (1.57 ng/g d.w.) detected at St.13. Spatial distributions of EDCs except for E2 in the sediments of Osaka Bay are shown in **Fig.2**. The ranges of EDC concentrations in sediments in the present study were similar with the ranges of them in Osaka Bay reported in other papers and also similar with detected concentrations in other areas as shown in **Table 1**. Higher concentrations of EDCs were observed in the inner bay and eastern side suggesting that EDCs were released from sewage effluents and introduced into coastal areas. As observed in Tokyo Bay (Isobe et al., 2001; Isobe et al., 2006), the concentrations of EDCs in the sediments of Osaka Bay appeared to decrease towards the open sea.

3.2. Measured inhibition rates

Some sediment extracts caused 100% inhibition rates even after 10 times dilution. Only after dilution by 300 times that less than 100% inhibition rates were observed in these extracts. Hence, sediment extracts were diluted 300 times before being subjected to the medaka ER binding assay. With this, the inhibition rates obtained ranged from 3 (station 1) to 99.7% (station 13). Simulation of the spatial distribution of inhibition rates (**Fig.3**) show that relatively higher values are observed in inner part of the bay especially in station 13 (off Sakai City) where almost 100% inhibition rate was detected. Meanwhile, less than 30% inhibition rates were observed in the southern part of the bay. In the assumption that inhibition rate is directly related to the estrogen receptor binding potencies as estrogenic potency, this implies that the estrogenic potency of sediments in the inner bay are consequently higher. In addition, this result coincides with the spatial distribution of EDCs in Osaka Bay and agrees with the assertion of Richard et al. (2004) that sediments with higher concentrations of EDCs also show higher estrogenic potency.

3.3. Estimated inhibition rates

The REP of each EDC was as follows: 4.69×10^{-4} for NP, 2.5×10^{-4} for OP, 3.29×10^{-6} for BP, 0.14 for E1 and 1.0 for E2. Total EEQs of sediments were calculated for stations (particularly 6, 13, 14, 17, 18, 22 and 24) with high measured inhibition rates. The calculated EEQs ranged from 0.004 (station 6) to 1.358 ng EEQ/g d.w. (station 13) as shown in **Table 2**. Based on these total EEQs, the inhibition rates were estimated and it ranged from 27.5 (station 6) to 48.8% (station 13). These results suggest that 49-66% of estrogenic potency of sediment extracts could be attributed to the 5 target EDCs. Based on Clemons et al. (1998), polyaromatic hydrocarbons (PAHs) are also estrogenic chemicals and PAH concentrations in sediments of Osaka Bay ranged from 6 to 7800 ng/g dry weight (not published our data) suggesting that PAHs are also main sources of estrogenic chemicals in the sediments.

Furthermore, according to the probit analysis of E2 concentration and inhibition rate in the medaka ER-α binding assay, most reliable estimated rate, a 50% inhibition rate (IC_{50}) corresponded to 6.2 pg EEQ/g sediment extract. Since the extracts were diluted by 300 times, an E2 equivalent estrogenic potency of 50% inhibition rate is, therefore, equivalent to 1.86 ng EEQ/g sediment extract. Based on reports of EEQ of sediment extracts from other coastal areas, which ranged from 0.0057 to 24.6 ng EEQ/g d.w. (Khim et al., 1999; Motegi et al., 2007; Tashiro et al., 2003; Thomas et al., 2004; Hashimoto et al., 2005; Petrovic et al., 2002), the estrogenic potency of sediment extracts of Osaka Bay was not very different. On the other hand, EEQ of riverine water, seawater and sewage effluent ranged from 0.001 to 31 ng EEQ/L (Motegi et al., 2007; Peck et al., 2004; Tashiro et al., 2003; Beck et al., 2006; Vajda et al., 2008; Thomas et al., 2004) showing that estrogenic potency of sediments are more than 1,000 times higher compared to water.

3.4. Contribution of each EDC to estrogenic potency

As shown in **Fig.4**, NP was a major contributor to the estrogenic potency ranging from 60 to 100% of these 5 EDCs estrogenic potency in stations 6, 14, 17, 18, 22 and 24. Moreover, share of E1 to the estrogenic potency ranged from 0 to 37 % in the same stations. Interestingly, E2 was the most important contributor to estrogenic potency in station 13 at 94 %. Grund et al. (2011) similarly found a sizeable contribution of NP to the estrogenic potency of sediment extracts from rivers with only 6% of the estrogenic potency attributed to BP and E1.

To explain variations in EDC contributions to estrogenic potency, relationship of EDC concentration to TOC of sediments was examined as shown in Fig.5. While estrogenic potencies of extracts did not show significant relationship with TOC, NP and OP concentrations in the sediments showed significant relationship ($p<0.05$) with TOC. NP, one of the main EDCs, with a high log K_{ow} (at 4.48) is possibly adsorbed strongly on organic particles. This could be the reason behind the higher contribution of NP to the estrogenic potency of sediments of Osaka Bay compared to the other potent EDCs.

3.5. Conclusions and recommendations

Since some EDCs are possibly transferred through the food chain, there is a great chance that EDCs detected in the sediments of Osaka Bay could also be accumulated by benthic fishes and could disrupt their endocrine systems. Hence, there is a need for management intervention to reduce the risks posed by relatively high concentrations of NP, E1 and E2 in the sediments of Osaka Bay.

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