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Biomagnification of endocrine disrupting chemicals (EDCs) by *Pleuronectes yokohamae*: Does *P. yokohamae* accumulate dietary EDCs?

Mohd Yusoff Nurulnadia[a,d], Jiro Koyama[b,*], Seiichi Uno[b], Haruna Amano[c]

[a] United Graduate School of Agricultural Sciences, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 890-0056, Japan
[b] Faculty of Fisheries, Kagoshima University, 4-50-20 Shimoarata, Kagoshima 890-0056, Japan
[c] School of Marine Biosciences, Kitasato University, 1-15-1 Kitasato, Minami, Sagamihara, Kanagawa 252-0373, Japan
[d] School of Marine and Environmental Sciences, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

HIGHLIGHTS

- This study aimed to investigate the bioaccumulation of selected EDCs through dietary route using *Pleuronectes yokohamae*.
- BMF values suggesting biomagnification not occurred which in contrast to high assimilation percentages.
- Glucuronidation was studied to investigate the possible metabolism and excretion of EDCs by *P. yokohamae*.

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ABSTRACT

We evaluated the potential for biomagnification of endocrine disrupting chemicals (EDCs) such as nonylphenol (NP), octylphenol (OP), bisphenol A (BP), and natural estrogens such as estrone (E1) and 17β-estradiol (E2) in a benthic fish, *Pleuronectes yokohamae*. The assimilation efficiencies (AE) of most EDCs ranged from 88 to 96% suggesting that they were efficiently incorporated and assimilated into *P. yokohamae*, except for NP (50%). However, the biomagnification factor (BMF) values were <1.0 suggesting that the compounds were not biomagnifying. Additionally, three of the target EDCs were not detected (BP, E1 and E2). Glucuronidation activity towards BP (11.44 ± 2.5 nmol/mg protein/min) and E2 (12.41 ± 3.2 nmol/mg protein/min) was high in the intestine suggesting that EDCs were glucuronidated prior to excretion into bile. Thus, we conclude that biomagnification of dietary EDCs is reduced in *P. yokohamae* because of effective glucuronidation.

1. Introduction

The presence of endocrine disrupting chemicals (EDCs) in the aquatic environment becoming a great concern due to the interference of these xenobiotics on the normal functioning of aquatic life endocrine systems. A number of estrogenic compounds have been detected in the environment include natural steroid hormones estrone (E1) and 17β-estradiol (E2). Industrial chemicals with estrogenic properties such as nonylphenol (NP), octylphenol (OP) (used as polyethoxylates in industrial detergents and emulsifiers) and bisphenol A (BP; used to produce epoxy resins and polycarbonate plastics) were detected as well (Lange et al., 2002). EDCs reach aquatic ecosystem mainly through sewage effluents thereby posing a potential threat to the aquatic organisms (Matthiessen and Sumpter, 1998).

The concentrations of nonylphenol (NP), octylphenol (OP), bisphenol A (BP), estrone (E1), and 17β-estradiol (E2) were measured in the sediment and the wild polychaete, *Paraprionospio* sp. from Osaka Bay. The result surprisingly showed the higher concentration of these EDCs in the *Paraprionospio* sp. than in the sediment (Koyama et al., 2013; Nurulnadia et al., 2014). Thus, we hypothesized that these compounds were being biomagnified in *Paraprionospio* sp. from the sediment. Furthermore, because polychaetes are an important component of the diet of benthic fish, the accumulated EDCs may be transferred into fish, and potentially humans, through the food chain. Thus, there is a need to understand the pathways of bioaccumulation and biomagnification of these compounds (Kristensen and Tyle, 1990; Lai et al., 2000; Wang et al., 1996).

* Corresponding author.
E-mail address: koyama@fish.kagoshima-u.ac.jp (J. Koyama).

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The aims of the present study were to examine 1) the assimilation efficiency, 2) biomagnifications, and 3) possible metabolism of EDCs in *Pleuronectes yokohamae*. Assimilation efficiency (AE) provides a measure of the fraction of ingested compounds that are incorporated into biological tissues (Jones et al., 2001; Olsen and Olsen, 2008). After organic contaminants are assimilated into fish tissues they may either biomagnify or be metabolized (Coldham et al., 1998). To investigate the biomagnification, *P. yokohamae* was exposed to EDCs through dietary route. EDCs structures often include phenols and aromatic groups (e.g. alkylphenols, phthalates and bisphenols), hence conjugation of polar moieties by phase II degradation enzymes is expected (Coldham et al., 1998; James, 2011; Thibaut et al., 1998; Thibaut and Porte, 2004; Yokota et al., 2002). The addition of glucuronyl group to the parent molecule is believed to increase the water solubility and facilitating the excretion (Giroud et al., 1998). Glucuronidation is one of the major metabolic pathways involved in the elimination of endogenous and exogenous compounds in fish (Dutton, 1980; Stegeman et al., 2010; Yokota et al., 2002). Therefore, to examine another possible process after assimilation of EDCs is metabolism by glucuronidation.

2. Materials and methods

2.1. Chemicals

EDCs standards were obtained from the Kanto Chemical Co. Inc., Japan (*p*-NP), Tokyo Chemical Ind. Co., Japan (OP), Nacalai Tesque, Inc. Kyoto, Japan (E2), Wako Pure Chemical Ind., Japan (BP, E1, E2-16,16,17-d3, and *p*-n-NP-d4), and Sigma Aldrich, United States of America (Bisphenol A mono-β-D-glucuronide, β-estradiol 17-β-D-glucuronide sodium salt, β-estradiol 3-β-D-glucuronide sodium salt, and UDPGA trisodium salt). Pesticide grade hexane, dichloromethane (DCM), diethyl ether, and florisil were purchased from Wako Pure Chemical Ind. Co., Japan. HPLC grade (methanol, isopropyl alcohol, ammonium acetate, and acetonitrile), high purity grade (magnesium chloride, potassium chloride, and sodium chloride), poisonous metal analysis grade (hydrochloric acid, perchloric acid, and nitric acid), Wako first grade (aprotinin from bovine lung, tris(2-amino-2hydroxymethyl)-1,3-propanediol, and chromium (III) oxide), and Wako special grade (2-phenoxyethanol and triethylamine) were also purchased from Wako Pure Chemical Ind. Co., Japan. Protein lysis solution CHAPs (3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate) and ethylenediaminetetraacetic acid disodium salt (EDTA 2NA) were purchased from Dojindo Molecular Technologies, Inc., Japan.

2.2. Test organism (P. yokohamae)

P. yokohamae is a benthic marine fish that is distributed from southern Hokkaido to Oita Prefecture in Kyushu, in the Yellow Sea, the Gulf of Bohai, and the northern part of the East China Sea (Sakamoto, 1984). *P. yokohamae* specimens were purchased from the Regional Government Aquaculture Center in Yamaguchi, Japan. They were reared under laboratory conditions for six months until they obtained the desired body size around 10 g (juvenile) and fed a commercial diet (Nippon Formula Feed Manufacturing Co., Japan).

2.3. Experimental design

2.3.1. Assimilation efficiency of EDC

We used an inert marker of chromium (III) oxide (0.5% in the diet) to estimate the assimilation efficiency of EDCs following the method described in Austreng et al. (2000). The diet was prepared by grinding 100 g of commercial fish diet with a pestle and mortar and mixing with 0.5 g of Cr (III) oxide. We then added sufficient water (10 mL per addition) so that the diet could be pelletized on a stainless tray using a syringe. The diet was dried under sunlight for 2 d and cut into the appropriate size for feeding. The mixture of EDCs was dissolved in diethyl ether and spiked into 50 g of the commercial diet containing 0.5% Cr (III) oxide in a stainless tray to aid the drying process. The spiked diet was left overnight in a draft chamber.

P. yokohamae were exposed to dietary EDCs and Cr (III) oxide in a glass aquarium (15 × 25 × 18 cm) with semi-static, well aerated water. The seawater was replaced once daily. The aquariums were placed in a heated water bath to maintain the temperature at 20 °C. Each aquarium was stocked with a single *P. yokohamae* (total of 9 aquaria) and the fish were starved for 2 d to clear the gut. After this, the fish in all tanks were fed the diet containing Cr (III) oxide for 3 d at a daily feeding rate 2% of the body weight. Feeding occurred twice daily and any food that was uneaten after 30 min was removed immediately. The diet was then switched to the EDC spiked diet (containing Cr (III) oxide) on days 4–6. During this period, feces were collected using siphon after naturally excreted by fish prior to feeding. The feces were then centrifuged at 760× g at 4 °C for 10 min. After removing the supernatant, the feces were stored in a freezer (−18 °C) until analysis. The body weight of individual *P. yokohamae* ranged from 17.0 g to 26.0 g at the beginning of the exposure experiments.

2.3.2. Biomagnifications of EDC

The concentration level was decided based on the measured EDCs in polychaete from Osaka Bay (Nurulnadia et al., 2014) as medium level, and 5 times lower and higher concentration to represent low and high levels, respectively. EDC standards were prepared for each compound at low, medium, and high concentrations. Each EDC was dissolved in diethyl ether and spiked into 50 g commercial diet (Nippon Formula Feed Manufacturing Co., Japan) in a stainless tray. The spiked diet was left overnight in a draft chamber. Sixteen aerated flow-through tanks (35 × 48 × 18 cm, 3 concentrations for each EDC and 1 control) were used for the exposure test of individual EDCs. Twenty-four of *P. yokohamae* were placed in each tank, including a control tank. The test water (30 L) was completely replaced 300 L/10 times/d with natural sand-filtered seawater. On day 0, three *P. yokohamae* individuals were sampled as a baseline. *P. yokohamae* in each tank were then administered one of the spiked diets at a daily feeding rate of 2% of their body weight for 14 d. Five fish in each tank were sampled on days 2, 4, 7 and 14. The average body weight of individual *P. yokohamae* was 2.95 ± 0.38 g and 3.51 ± 0.49 g, for the control and exposure groups, respectively, at the beginning of the experiment. Non-consumed food residue was removed daily. During sampling, *P. yokohamae* were anaesthetized in 0.05% 2-phenoxyethanol. After measuring the body length and weight, the fish were stored in a freezer (−18 °C) until EDC analysis.

2.4. Chemical analysis

2.4.1. Quality assurance

The recovery test was performed using the fish diet spiked with 100 ng/g of each EDC standard and both external and internal standards (*p*-n-NP-d4 and E2-16,16,17-d3). The EDC standard extraction and measurement were performed using the procedures described above. Mean percent recovery, calculated based on the amount added and recovered, was between 60 and 104%. All analyses were performed in triplicate (n = 3) with the error of all analysis within ±25% (Table 1).

Table 1
Precursor and daughter ions of EDCs, limit of detection (LC–MS/MS–ESI) and extraction efficiency (%) in feces, diet & fish tissue and seawater (n = 3).

EDCs	Precursor ions, m/z (amu)	Daughter ions, m/z (amu)	Limit of detection[a]		
			Feces (ng/g)	Diet & fish tissue (ng/g)	Seawater (ng/mL)
NP	219.0	132.9	1.70 ± 0.1	1.89 ± 0.4	0.15 ± 0.09
OP	205.0	132.9	1.01 ± 0.3	1.17 ± 0.5	0.01 ± 0.005
BP	227.1	211.8	1.35 ± 0.1	1.76 ± 0.9	0.22 ± 0.1
E1	269.1	145.0	1.23 ± 0.2	1.13 ± 0.7	0.23 ± 0.03
E2	271.1	144.9	0.50 ± 0.2	0.22 ± 0.1	0.12 ± 0.09

Extraction efficiency (%)

	Homogenate tissue		Feces	Diet & fish tissue	Seawater
	Intestine	Liver			
NP	n.a[b]	n.a	61 ± 7.0	60 ± 7.4	60 ± 6.7
OP	n.a	n.a	95 ± 6.5	92 ± 6.4	100 ± 5.4
BP	66 ± 14	70 ± 13	73 ± 6.0	60 ± 5.8	60 ± 3.9
E1	n.a	n.a	107 ± 4.5	103 ± 4.1	104 ± 5.8
E2	75 ± 15	72 ± 24	94 ± 3.5	90 ± 5.2	92 ± 9.0

[a] Detection limit using 0.3 g diet, fish tissue, feces and 1 L seawater.
[b] Not available.

2.4.2. Chromium analysis in diet and feces

The digestion of Cr (III) to Cr (VI) was accomplished by, adding 5 mL concentrated nitric acid to a beaker containing either the spiked diet (0.3 g) or feces (0.01–0.017 g). The beaker was then covered with a watch glass and refluxed on a hotplate at 60–220 °C. After addition of 3 mL perchloric acid, the sample was covered with a watch glass and gradually heated on a hotplate (from 220 to 330 °C) for 40 min. The absorbances of samples were measured using a UV–visible Spectrophotometer (Shimadzu 1600 UV–Visible) at a wavelength of 350 nm. EDCs were analyzed as described in section 2.5 EDC measurement using LC–MS/MS–ESI.

2.4.3. EDC analysis in P. yokohamae*, diet and feces*

EDC analyses were performed as described in Nurulnadia et al. (2014). After addition of external standard of *p*-n-NP-d4, the target chemicals in the freeze-dried whole *P. yokohamae* (homogenate weight ranged from 0.78 to 3.05 g), diet (0.3 g), and feces (0.01–0.017 g) were ultrasonically extracted twice by mixing with 20 mL of methanol for 15 min. After centrifugation (4 °C, 760× g, 10 min), the organic layer was collected and added to 3 mL mili-Q water and 3 mL hexane. The lipid was separated from methanol by shaking into 20 mL hexane three times. The hexane layer was discarded and the methanol was collected and combined. The methanol was mixed with 50 mL of 5% sodium chloride and 0.2 mL concentrated hydrochloric acid. The mixture of solvents was extracted twice with 20 mL dichloromethane and concentrated to 0.5 mL using a rotary evaporator and a gentle stream of N_2. The concentrated extract was then replaced with hexane. Clean up was performed in a glass pipette (6 mm i.d. × 17 cm) filled with florisil (containing 3% water) and prewashed with hexane. The target chemicals were eluted with 50 mL hexane-isopropyl alcohol (9:1, v/v). The eluate was then concentrated by drying using a rotary evaporator and a gentle stream of N_2. Last, an internal standard (E2-16,16,17-d3) and acetonitrile were added to make a final volume of 0.2 mL before LC–MS/MS measurement. EDC extraction from seawater samples was carried out within 24 h of collection. After addition of the external standard (*p*-n-NP-d4), a liquid–liquid extraction was performed on 1 L of glass-fiber (GF/C Whatman)-filtered sample using 100 mL hexane and shaking in a separating funnel. After complete separation, the hexane layer was collected while the aqueous portion was subjected to a second extraction. The first and second extracts were combined and dehydrated using sodium sulfate anhydrous. Further extraction was performed using 100 mL DCM and the layer was gathered into a rotavapor flask. The extracted solvent was then concentrated by drying under a gentle stream of N_2 at 40 °C, then made up to 0.5 mL with acetonitrile and an internal standard (E2-16,16,17-d3). Samples were then subjected to LC–MS/MS measurement.

2.4.4. Enzyme analysis in the intestine and liver of P. yokohamae

Prior to analysis, *P. yokohamae* individuals were starved for 1 d to empty the digestive tract. Each fish was then anaesthetized with 0.05% 2-phenoxyethanol, the body length (mean: 8.94 ± 0.2 cm) and weight (mean: 8.19 ± 1.1 g) were measured, and the intestine and liver tissues were dissected out. The mean weight of the intestine and liver tissues was 0.343 ± 0.1 and 0.247 ± 0.03 g, respectively. Each tissue was homogenized in 4 volumes of a solution containing 0.15 M potassium chloride and 1 mM EDTA 2Na in ice. The homogenate was centrifuged at 4 °C and 10,000× g for 10 min to obtain crude microsomes. Rapid preparation of the microsomal fraction was essential to prevent the loss of UGT activity. Enzyme analysis was performed following the method in Yokota et al. (2002) with modifications. Glucuronide/UGT activity toward BP and E2 (substrates) was assayed in 400 μL of 100 μL crude microsomes with 5 μL 0.01% protein lysis CHAPs, 250 μL of buffer 0.1 mM Tris–HCl containing 5 mM magnesium chloride (pH 7.4), 5 μL substrate (BP and E2), and 40 μL of 5 mM UDPGA trisodium salt at 30 °C over a 30 min incubation period. We used a target concentration of 5 μg/mL for each substrate (BP and E2) due to the sensitivity of the HPLC measurements. The enzyme activity was stopped by boiling in water for 5 min and the reaction products were filtered using a disposable disk (Whatman syringe filter pore size 0.2 μm, Sigma–Aldrich, USA) and analyzed by HPLC using a LaChromUltra system (Hitachi Tokyo, Japan) equipped with a fluorescence detector (L-2485U). Substrate-glucuronide peaks were estimated using their respective authentic glucuronide standards. The filtered samples (10 μL) were injected by an autosampler (L-2200U) into an Inertsil ODS-3 column (2.1 mm i.d. × 100 mm, 3.0 μm particle size, GL Sciences Inc., Japan) that was maintained at 40 °C and eluted with a gradient program of acetonitrile (mobile phase A) and 10 mM ammonium acetate (mobile phase B) at a constant flow rate of 0.6 mL/min: 5 (A) and 95% (B) for the first 10 min, 50 and 50% from 10 to 16 min, and 5 and 95% from 16 to 22 min. The fluorescence excitation and emission wavelengths were 275 and 308 nm, respectively. The microsomes were activated by incubation with 0.01% CHAPs for 30 min at 0 °C. The protein concentration was determined using a Bio-Rad Protein Assay with bovine serum albumin as a standard and measured on a microplate

reader (MTP-32, Corona Electric Co. Ibaraki, Japan) at a wavelength of 630 nm.

2.5. EDC measurement using LC–MS/MS–ESI

EDC measurements were performed following the method in Nurulnadia et al. (2013). We measured the concentration of EDCs by LC–MS/MS using an Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an API-2000 triple stage quadruple mass spectrometer equipped with an ESI source (Applied Biosystems, USA). The ESI was used in negative mode. The source parameters for MS detection were optimized with the quantitative function of Analyst (Version 1.4.1, Applied Biosystems) equipped with API-2000. The optimized parameters were: curtain gas (40 psi), turbo gas (80 psi), and auxiliary gases (80 psi) using nitrogen, CAD gas, 5 psi; ionspray voltage – 4500 V; and turbo temperature, 400 °C. Ionization and fragmentation settings were optimized by directly injecting the solution containing EDCs with MS/MS measurements performed in the multiple reaction monitoring (MRM) mode. The analysis used a reverse-phase Extend C-18 column (2.1 mm i.d. × 100 mm, 3.5 μm particle size, Agilent) kept at 40 °C. The mobile phase consisted of solvents A (acetonitrile) and B (0.01% triethylamine, (v/v)). The analytes were eluted using the following gradient program:

An increase from 20 to 95% of solvent A over a 15 min period, then 10 min at 95% solvent A. The solvent A flow was increased during column washing to 500 μL/min within 0.1 min and maintained for 2 min (not flowing to the ESI detector using an LC–MS/MS equipped with a 6-port switching valve system). After washing the column, the concentration of solvent A was changed back to the initial ratio within 0.1 min and held for 8 min at a flow rate of 500 μL/min until the next injection. The detection limit for each EDC is shown in Table 1.

2.6. Data analysis and statistical analysis

The assimilation efficiency (%) was estimated using the formula: 100 – [100 × (Absorbance of Cr in diet/Absorbance of Cr in feces) × (EDC in feces/EDC in diet)]; where Cr represents the chromium absorbance/g in diet or feces, and EDC represents the EDC concentration on a dry weight basis.

BMFs were calculated as the ratio of the concentration in the organism to the diet at steady state as follows: BMF = (EDC concentration in *P. yokohamae* at steady state)/(EDC concentration in spiked diet) on a wet weight basis.

Glucuronidation/UGT activity was calculated by measuring the concentration of glucuronide substrate (nmol) produced per mg protein per min incubation (nmol/mg protein/min).

Statistical analyses were conducted using Minitab 16. We used Friedman and Two-way ANOVA test to test the relationship of body burdens to dose and time of exposure.

3. Results and discussion

3.1. Water parameters

The mean water temperature, dissolved oxygen concentration, pH, and salinity were 21.7 ± 0.9 °C, 5.86 ± 1.3 mg/L, 7.80 ± 0.2, and 33.3 ± 0.2 ppt, respectively, during the assimilation efficiency experiment. Mean water temperature, dissolve oxygen, pH, and salinity were 26.7 ± 0.9 °C, 6.67 ± 0.4 mg/L, 8.28 ± 0.1, and 28.0 ± 0.2 ppt, respectively, during the biomagnification experiment.

Table 2
Mean ± SD[a] values of Cr (VI) and EDCs in the spiked diet and feces, ng/g dry weight (n = 3) and assimilation efficiency in percentage.

EDCs	Abs[b] Cr/diet weight (g d.w.[c])	Abs Cr/feces weight (g d.w.)	Diet	Feces	Assimilation efficiency (%)
NP	2.65 ± 0.2	12.0 ± 2.7	1200 ± 190	2790 ± 1120	49.6 ± 9.4
OP	2.65 ± 0.2	12.0 ± 2.7	479 ± 110	253 ± 44	87.9 ± 3.5
BP	2.65 ± 0.2	12.0 ± 2.7	424 ± 21	105 ± 73	93.9 ± 5.4
E1	2.65 ± 0.2	12.0 ± 2.7	136 ± 8.7	42 ± 26	92.4 ± 6.2
E2	2.65 ± 0.2	12.0 ± 2.7	552 ± 5.0	78 ± 65	96.4 ± 3.6

[a] SD – standard deviation.
[b] Abs – absorbance.
[c] d.w. – dry weight.

3.2. Assimilation efficiencies (AE) of EDCs

Cr (III) oxide was used as the inert marker in this study to estimate digestibility because it could be incorporated homogenously into the feed, analyzed accurately at low concentrations, is indigestible, and does not affect metabolism but passes through the gastro-intestinal tract with dietary nutrients (Austreng et al., 2000). The absorbance of Cr (VI) in the spiked diet and feces, and concentration of each EDC in the diet and feces are given in Table 2. The moisture content of feces was in the range of 3.99–10.2% (mean: 7.07 ± 2.6%) but moisture content of diet was not measured as the fishes were fed dry diet.

The assimilation efficiency of each EDC is given in Table 2. *P. yokohamae* were least efficient at assimilating NP (49.6 ± 9.4%) and most efficient at assimilating E2 (96.4 ± 3.6%). The AE values for OP, BP, and E1 were >88% suggesting that *P. yokohamae* efficiently assimilates the target EDCs, except for NP. EDCs level in seawater was below detection limit suggest EDCs not released from the diet into seawater (Table 1).

We compared the AE values for NP in *P. yokohamae* to fathead minnows. After exposing fathead minnows to dietary NP, Pickford et al. (2003) found very low percentage of NP in the feces indicating assimilation from the gastrointestinal tract was almost complete. In contrast, *P. yokohamae* assimilated a much lower percentage of NP (49.6 ± 9.4%). Liang et al. (2007) proposed that K_{ow} (octanol–water partition coefficient) would enhance biotransformation and decreased gut assimilation of PAH in tilapia. Thus, fish situated at a high trophic level in the food web have the ability to rapidly metabolize PAHs and/or reduce their assimilation. The decrease of NP has also been reported in the prey–predator relationship between the microalga *Isochrysis galbana* previously incubated with NP and the crustacean *Artemia fransiscana* (Correa-Reyes et al., 2007). *Isochrysis* bioconcentrated NP 6940 times, and only traces of NP could be found in *Artemia* after 56 days feeding on the alga; it was concluded that *Artemia* metabolized almost all NP ingested and reduce the assimilation of NP. In the present study, K_{ow} of NP (4.48) is the highest among studied EDCs, hence attribute to biotransformation/metabolization in *P. yokohamae* which reduce the assimilation percentage. The percentage of OP in rainbow trout feces was low (0.2%, ~500 times lower than diet), thus the estimated AE was 99.8% (Pedersen et al., 2003). This value is similar to the AE for OP in our study (87.9 ± 3.5%), suggesting that both rainbow trout and *P. yokohamae* can efficiently assimilate OP. To our knowledge, this is the first study to report the assimilation efficiency for BP, E1, and E2 in an aquatic organism.

The AE values suggest that *P. yokohamae* easily assimilates the target EDCs through dietary exposure. Thus, there is potential for biomagnification of EDCs that are assimilated into *P. yokohamae* via the dietary route.

Table 3
Mean EDCs concentration, ng/g dry weight, in the spiked diet and fish tissue at steady state, and BMF±SD[a] values measured from ratio of EDCs concentration in fish tissue to diet, n = 3.

	Diet	Fish tissue	BMF
NPL	13,600 ± 154	270 ± 12	0.020
NPM	49,700 ± 114	335 ± 10	0.007
NPH	259,000 ± 40	449 ± 3.3	0.002
OPL	55.4 ± 0.8	6.19 ± 0.06	0.112
OPM	309 ± 0.6	8.96 ± 0.05	0.029
OPH	2920 ± 7.9	19.6 ± 0.72	0.007

[a] SD – standard deviation.

3.3. Biomagnification factors (BMF) of EDCs

The concentrations of each EDC in the spiked diet are given in Table 3. NP, OP, and BP were detected in the control diet at concentrations of 28.3 ± 1.8, 3.02 ± 1.2, and 2.68 ± 1.3 ng/g, respectively. Pellets of commercial diet generally consist of 36–52% fish meal from wild catches (Olsen, 2011). Thus, it was expected that the control diet would contain low concentrations of EDCs. EDCs level in seawater was below detection limit suggest EDCs not released from the diet into seawater (Table 1).

The moisture content of *P. yokohamae* was in the range of 65.5–73.4% (mean: 70.1 ± 5.4%). The concentration of NP and OP in *P. yokohamae* is shown in Fig. 1. However, BP, E1, and E2 were not detected. These compounds were probably degraded and biotransformed into a polar form to facilitate elimination (Klaassen, 2001; Marx, 1974). In fish, the majority of organic xenobiotics are biotransformed by oxidation in phase I reaction and glucuronide conjugation in phase II reaction (Melancon and Lech, 1976; Schlenk et al., 2008; Varanasi and Gmur, 1981). Because biotransformation of EDCs is one of the primary factors determining their tissue distribution in fish (Lech, 1974), the presence of a polar compound like glucuronide will reduce bioaccumulation/biomagnification (Van den Berg et al., 2003) as we discussed below.

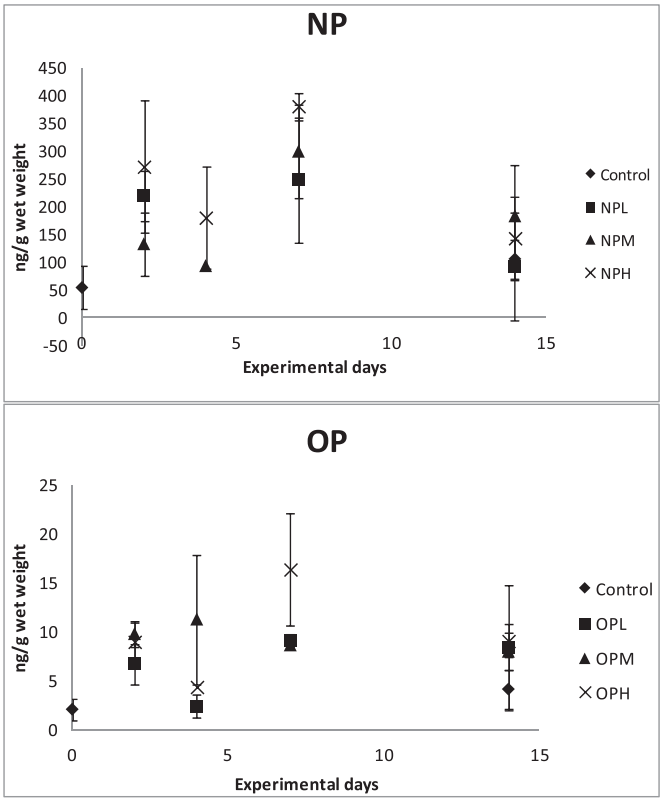


Fig. 1. Accumulation of NP and OP (ng/g wet weight) in *P. yokohamae* in control, low, medium, and high concentration exposure groups. Error bars represent the standard deviation (n = 3). NP in control fish at day 0 and 14 were 53.6 ± 21 and 79.0 ± 35 ng/g w.w., respectively, and OP were detected at day 0 and 14 with 2.05 ± 0.9 and 4.11 ± 1.9 ng/g w.w., respectively. (NPL-nonylphenol low; NPM-nonylphenol medium; NPH-nonylphenol high; OPL-octylphenol low; OPM-octylphenol medium; OPH-octylphenol high concentration).

On day 0, NP and OP were detected in *P. yokohamae* at concentrations of 53.6 ± 21 and 2.05 ± 0.9 ng/g wet weights (w.w.), respectively (Fig. 1). As expected, NP and OP were also detected in control fish as *P. yokohamae* accumulated very low concentrations of these compounds from the control diet during the acclimation period. The concentration of NP and OP increased rapidly from day 0 and the highest concentration was observed on day 7 for most of the exposure groups. The maximum concentrations of NP in the low, medium, and high exposure groups were 248 ± 56, 299 ± 85, and 380 ± 25 ng/g w.w., respectively, whereas the maximum concentrations of OP in the low, medium, and high exposure groups were 9.02 ± 6.5, 11.3 ± 3.1, and 16.3 ± 5.7 ng/g w.w., respectively. Thus, the concentrations of both NP and OP appeared to plateau on day 7, so these concentrations were used for calculation of the BMF. There were no significant difference ($p > 0.05$) between accumulation level of NP and OP to the dose given and time of exposure.

BMFs were calculated using the concentration of NP and OP at day 7 of each group to the measured concentration of the respective compound in the spiked diet (Table 3). BMF values of both EDCs in each group were much less than 1, suggesting that these compounds were not biomagnified by *P. yokohamae* from the diet (Berntssen et al., 2007). *P. yokohamae* were exposed to high, medium, and low concentrations of each EDC and the results showed BMF values decreased with increases of NP or OP concentrations in the spiked diet. Since BP, E1, and E2 were not detected (below detection level), BMF value was not measured.

Consistent with our observations, the BMF values for NP and OP in previous studies (shown in Table 4) were generally < 1, suggesting that biomagnification does not occur. However, three BMF values were > 1. In these instances, the high values may result from bioconcentration (water) and bioaccumulation (prey). In contrast, the low BMF values in our study may be a function of diet as the only route of exposure.

The high assimilation efficiency for EDCs was in contrast to the low biomagnification factor in *P. yokohamae* suggesting that the EDCs have been metabolized (Müller et al., 1998; Pickford et al., 2003; Pottenger et al., 2000). The gastrointestinal tract plays a role in metabolizing organic contaminants by fish and can substantially reduce their bioaccumulation/biomagnification (Kleinow and James, 2001; Nichols et al., 2004). To test whether this was the case, we evaluated the level of EDCs metabolism in the intestine and liver of *P. yokohamae*.

3.4. Glucuronidation/UGT activity of BP and E2 in P. yokohamae

We chose BP and E2 for determination of glucuronidation/UGT activity because of the commercial availability of glucuronide standards for these EDCs. The chromatogram of U-HPLC analysis of BP and E2 obtained *in vitro* from *P. yokohamae* intestine and liver microsomes are shown in Fig. 2. Parent BP was eluted at 10.5 min. The retention time of BP glucuronide (BPG) was identified by referring to the standard of bisphenol A mono-β-D-glucuronide which eluted at 6.6 min. Meanwhile, parent E2 was eluted at 8.9 min, and the retention time of E2 glucuronides (E2G) were identified by referring to the standard of β-estradiol 3-(β-D-glucuronide) sodium salt (E2G-3) and β-estradiol 17-(β-D-glucuronide) sodium

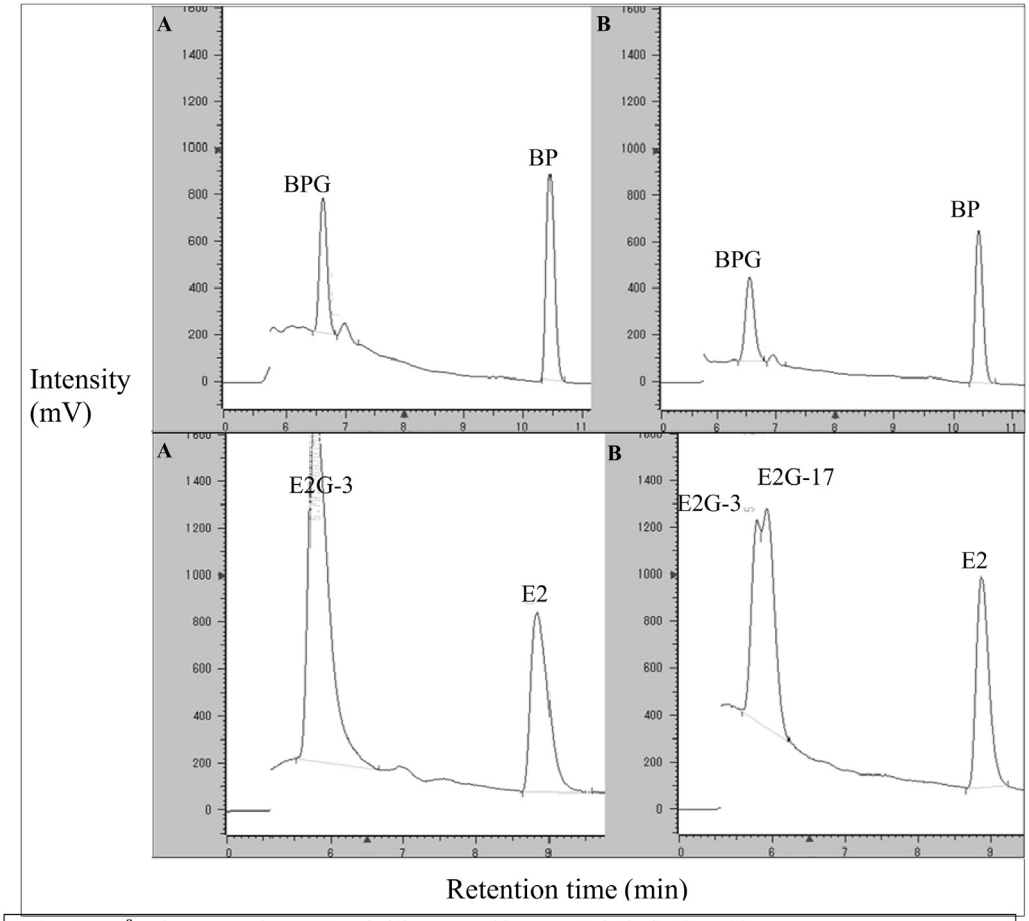
Table 4
BMF of NP and OP based on the ratio in predator to prey.

Predator	Prey	BMF		Location	Reference
		NP	OP		
Food web analysis		0.196	0.277	Tokyo Bay, Japan	Takeuchi et al., 2009
Ghost shrimp	Benthic invertebrates	0.2	n.a	California estuaries, USA	Diehl et al., 2012
Goby liver	Benthic invertebrates	0.3	n.a	California estuaries, USA	
Snail	Oyster	1.3	2.0	West coast of Taiwan	Cheng et al., 2006
Herring gull	Various fish	1.02	n.a	Bohai Bay, China	Hu et al., 2005
Polychaete	Diet	0.001	0.005–0.009	Exposure test	Nurulnadia et al., 2013
P. yokohamae	Diet	0.002–0.02	0.007–0.112	Exposure test	This study

n.a – Not available.

salt (E2G-17) which eluted at 5.8 and 5.93 min, respectively.

Our results suggest that BP and E2 were glucuronidated by microsomes prepared from the intestine and liver of *P. yokohamae* after activation with UDP-glucuronic acid and incubation. There was no significant difference ($p > 0.05$) in the UGT activities towards BPG and E2G-3 between intestinal and liver microsomes. The UGT activity towards BP was higher in the liver (26.5 ± 18 nmol/mg protein/min) than in the intestine (11.4 ± 1.2 nmol/mg protein/min) (Fig. 2). Two conjugates of E2 were found in the intestine, E2G-3 (2.40 ± 1.2 nmol/mg protein/min) and E2G-17 (12.4 ± 3.2 nmol/mg protein/min), but only E2G-3 (12.7 ± 0.6 nmol/mg protein/min) was detected in the liver.



Mean±SD[a] microsomal UGT activities, nmol/mg protein/min (n = 4)

Microsome	BPG	E2G-3	E2G-17
Intestine	11.4±1.2	2.40±1.2	12.4±3.2
Liver	26.5±18	12.7±0.6	

Fig. 2. UGT activities in *P. yokohamae* and chromatograms of BP (upper) and E2 (lower) in liver (A) and intestine (B) tissue following activation by UDP-glucuronic acid and incubation ([a]SD–standard deviation).

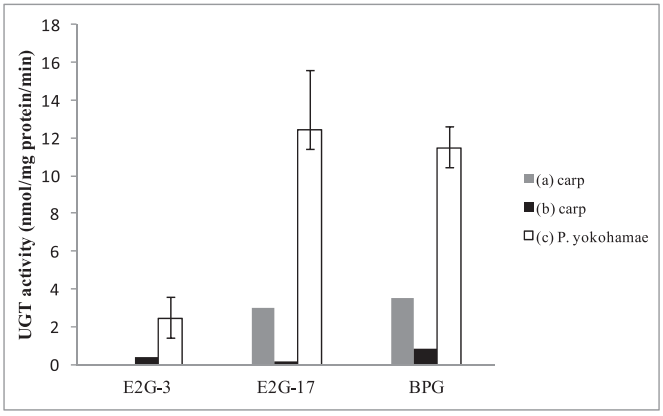


Fig. 3. Microsomal UGT activities toward BP and E2 in the intestine of carp and *P. yokohamae* (a) Yokota et al., 2002, (b) Daidoji et al., 2006, (c) this study (n = 4).

The majorities of organic xenobiotics are biotransformed by phase I (oxidation) and phase II (glucuronide) conjugation (Melancon and Lech, 1976; Schlenk et al., 2008; Varanasi and Gmur, 1981). However, the direct glucuronidation of EDCs and estrogen more frequently reported in fish (Coldham et al., 1998; James, 2011; Thibaut et al., 1998; Thibaut and Porte, 2004; Yokota et al., 2002). Thus, we measured intestinal UGT activity to evaluate the occurrence of glucuronidation in *P. yokohamae*. UGT activities toward BP and E2 in the intestine of carp and *P. yokohamae* are shown in Fig. 3. High microsomal UGT activity in *P. yokohamae* suggests they were metabolizing BP and E2. The higher level of UGT activity in the intestine of *P. yokohamae* compared to carp (Daidoji et al., 2006; Yokota et al., 2002) may reflect species differences in the ability to metabolize BP and E2 (Lindholst et al., 2001, 2003).

After glucuronidation in the intestine, the glucuronidated BP and E2 were transferred to the liver and then excreted into the bile. Meanwhile, the glucuronidated BP and E2 in the liver also excreted via the bile shortly after intake or can go into circulation before being transported to the liver to be conjugated and excreted via the bile. The bile content was then transferred back to intestine, where it is excreted in the feces (Houtman et al., 2004). Thus, UGT activity appears to reduce biomagnification of these EDCs in *P. yokohamae* as our target compounds were the parent EDCs. Additionally, glucuronidated BP and E2 were expected to be rapidly eliminated due to their higher solubility. According to our study, metabolisms of BP and E2 into BPG and E2G have verified it could be one of reason for both compounds did not biomagnified in *P. yokohamae*.

The other EDCs (NP and OP) were also reported to be glucuronidated in fish (Thibaut et al., 1998; Ferreira-Leach and Hill, 2001; Pedersen and Hill, 2000). Thus, further test are required to verify the glucuronidation/UGT activity of NP and OP in *P. yokohamae*. However, to our knowledge no study reported on glucuronidation of E1 in fish.

4. Conclusion

The potential for biomagnification of EDCs through the food chain was investigated in a benthic fish, *P. yokohamae*. The high assimilation efficiency for EDCs (ranged from 50 to 96%) suggests that *P. yokohamae* are able to uptake the target EDCs from the dietary route. However, BMF values were very low (<1.0) and indicate that biomagnification of EDCs does not occur. Increased glucuronidation activity of BP and E2 in the intestine and liver suggests that the compounds are instead metabolized and eliminated. But, further test are required to verify the glucuronidation of NP and OP in *P. yokohamae*.

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